Universidade de Aveiro Departamento de Química
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Caracterização dos mecanismos moleculares subjacentes à caquexia associada ao cancro da cabeça e pescoço

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção de grau de Mestre em Bioquímica, ramo Bioquímica Clínica, realizada sob a orientação científica do Doutor Rui Miguel Pinheiro Vitorino, Investigador Principal do Departamento de Ciências Médicas da Universidade de Aveiro e da Doutora Rita Maria Pinho Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho à minha família por todo o apoio.

o júri

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palavras-chave Cancro da cabeça e pescoço, caquexia, catabolismo muscular, biomarcadores, proteómica da urina

resumo O cancro da cabeça e pescoço é o sexto tipo de cancro mais comum no mundo, com 630.000 novos casos diagnosticados e mais de 350.000 mortes registados anualmente. Os pacientes com cancro da cabeça e pescoço apresentam, geralmente, uma perda de peso acentuada devida, principalmente, à perda de massa muscular. Estes sinais de catabolismo estão associados a uma síndrome que se designa de caquexia. Esta síndrome compromete o prognóstico e a qualidade de vida do paciente oncológico. Apesar da sua importância clínica, não existem opções terapêuticas eficazes para o tratamento da caquexia associada ao cancro, o que pode ser justificado, pelo menos em parte, pela dificuldade em diagnosticar os pacientes com caquexia.

O objetivo desta dissertação foi caracterizar o fenótipo de pacientes com cancro da cabeça e pescoço com e sem perda de peso corporal e identificar possíveis biomarcadores de caquexia em fluidos biológicos para melhorar o diagnóstico e tratamento destes pacientes. Para o efeito estudaram-se 30 pacientes do sexo masculino com cancro da cabeça e pescoço que foram agrupados com base no índice de risco nutricional (NRI, *nutritional risk index*) em dois grupos: com caquexia (NRI<97,5; n=14) e sem caquexia (NRI>97,5; n=16). A análise de parâmetros séricos destacou a importância da inflamação para a caquexia e a sua associação com o estadio da doença. Os níveis séricos elevados de miostatina observados em doentes com caquexia suportam a ocorrência de catabolismo muscular neste grupo de doentes. Mais ainda, verificou-se uma correlação negativa entre os valores de proteína reativa C (CRP) e a grelina, o que suporta a potencial aplicação terapêutica dos agonistas de grelina no tratamento de doentes com perda de peso e valores de CRP superiores a 30 mg/dL. A análise do proteoma da urina permitiu identificar 18 proteínas moduladas pela caquexia, das 520 das proteínas identificadas por GeLC-MS/MS, sendo de destacar as proteínas envolvidas na inflamação CRP, *alpha chain of C4-binding protein*, e argininosuccinato sintase. No futuro será importante validar o valor de diagnóstico destas proteínas para o diagnóstico da caquexia associada ao cancro da cabeça e pescoço e, eventualmente, de outros tipos de cancro.

keywords Head and neck cancer, cachexia, muscle wasting, biomarkers, urine proteomics

abstract Head and neck cancer (HNC) is the sixth most common cancer worldwide, with estimated 630,000 new patients diagnosed annually, resulting in more than 350,000 deaths every year. Patients with HNC usually have large weight loss often associated with cachexia, a syndrome mainly characterized by muscle mass loss. Cachexia has been underdiagnosed in the set of HNC, resulting in increased rates of morbidity and impaired quality of life of patients. There are no effective therapeutic options for the management of this syndrome, which can be justified, at least in part, by its misdiagnosis.

The objective of this dissertation was to characterize the body wasting phenotype of HNC patients and identify putative biomarkers to improve the early diagnosis and management of HNC cachexia. Thirty male patients were enrolled in this study and were assigned to one of two groups based on the nutritional risk index: HNC (NRI>97.5; n=16) or HNC+ cachexia (NRI<97.5; n=14). Targeted and nontargeted proteomic approaches were applied to the analysis of blood-derived serum and urine. Data highlighted the contribution of inflammation to the development of cachexia given by the high levels of C-reactive protein (CRP), IL-6 and TNF-α, which were associated to advanced stages of disease. The high circulating levels of myostatin seem to corroborate the occurrence of muscle wasting. The negative correlation observed between CRP and ghrelin suggests that HNC cachectic patients with CRP levels higher than 30 mg/dL may benefit from therapy with ghrelin agonists. Urine proteomics allowed the identification of 18 proteins modulated by cachexia, within the 520 distinct proteins identified by GeLC-MS/MS, such as alpha chain of C4-binding protein, CRP and argininosuccinate synthase, which emphasizes the contribution of inflammation to cachexia pathogenesis. Future studies will be important to validate the diagnosis value of these proteins for the management of cachexia in HNC and, eventually, in other types of cancer.

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Abbreviations

ACN	Acetonitrile
ActA	Activin-A
ActRIIB	Act receptor type-2B
Akt/PKB	Protein Kinase B
BMI	Body mass index
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EGF	Epidermal growth factor
EBV	Epstein-Barr virus
FOX-O	Transcription factors forkhead
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gp130	Glycoprotein 130
HGF	Hepatocyte growth factor
HNC	Head and neck cancer
HNSCC	Head and Neck Squamous cell carcinoma
HPV	Human papilloma virus
HSV	Herpes Simplex virus
IAA	Iodoacetamide

IFN-γ	Interferon-gama
IGF-1	Insulin-like growth factor 1
IGF1R	IGF1 receptor
IkB	Inhibitor of kappa B
Ikk	IκB kinase
IL	Interleukin
IRS-1	Insulin receptor substrate 1
JAK	Janus Kinase
LC-MS/MS	Liquid chromatography–Tandem mass spectrometry
M	Distant metastases
MAPK	Mitogen-activated protein kinase
MMP	Metalloproteinase
Mstn	Myostatin
mTOR	Mammalian target of rapamycin
MuRF1	Muscle RING finger proetein 1
MUST	Malnutrition Universal Screening
N	Regional nodal spread
NF	Nuclear factor
NRI	Nutrition Risk Index
PI3K	Phosphoinositide 3-kinases
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodiu dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SOCS3	Suppressor of cytokine signaling 3
SSA	Serum amyloid-A
RT	Radiation therapy
T	Primary tumor size
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
UPS	Ubiquitin-proteasome system
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

Cancer is responsible for one in eight deaths worldwide, it is a leading cause of death in more and less economically developed countries (1,2). These numbers are expected to increase due to the growth and aging of the population, particularly in less developed countries. For this increase contributes lifestyle behaviors that are known to rise cancer risks, such as smoking, poor diet, physical inactivity, and reproductive changes (including lower parity and later age at first birth) (2). According to GLOBOCAN, 18,1 million new cases of cancer and 9,6 million cancer deaths worldwide were estimated for 2018 (3). It is anticipated that these numbers will increase in the future. According to the World Health Organization (WHO), from 2018 to 2040 the incidence of cancer will increase from 18,1 million to 29,5 million (4).

1.1. Head and Neck Cancer: facts and risk factors

Head and neck cancer (HNC) is a malignant neoplasm that can be found in patients all over the world (5). HNC is the sixth most common cancer worldwide, with estimated 630,000 new patients diagnosed annually resulting in more than 350,000 deaths every year (6). In overall, the incidence of HNC cancer in males is 3.4% and mortality is 2%. In females, these values are lower, the incidence is 1.6% and mortality is 1% (7). For men aged between 60 and 80 years old, HNC is a common cause of death, worldwide; however, countries such as Melanesia, South Asia, parts of France, and much of Eastern Europe and the former Soviet republics present the highest rates (6). According to GLOBOCAN, the incidence of HNC will be higher in 2030 compared to 2012. The incidence of lip and oral cavity cancer in men is expected to increase worldwide, but the difference will be greater in less developed countries (more of 50%). It is also predicted the increase of larynx and nasopharynx cancer incidence by 2030 in the least developed countries, which is expected to be even greater for larynx cancer than oral cancer (estimated at 69.5%). Table 1 highlights the incidence of the 4 types of HNC expected for 2030, the values for 2012 represent an estimate of patients diagnosed in this year (8).

Table 1. *Estimates of HNC incidence in men, in different locations, up to 2030. Differentiated estimates between less and more developed countries are presented (adapted from* (8)*).*

	World		**More developed countries**		**Less developed countries**	
Year / Cancer type	2012	2030	2012	2030	2012	2030
Lip and oral cavity	198,975	298,854	68,042	82,780	130,933	208,833
Other pharynsx	115,131	175,171	44,400	52,076	731	116,737
Larynx	138,102	216,349	50,730	62,575	87,372	148,138
Nasopharynx	60,896	84,943	5,071	5,771	55,825	83,397

Squamous cell carcinoma (HNSCC) is the most common type of HNC, representing approximately 90% of the cases of HNC (9). This type of cancer affects the upper paranasal sinuses, nasal cavity, oral cavity, salivary gland, pharynx, and larynx (Figure 1) (10). HNSCC is characterized by phenotypic, etiological, biological and clinical heterogeneity (11). The most common symptoms are sore throat, hoarseness, swallowing difficulties and oral ulceration, and it depends on the location and stage of the tumor (12). Tumors in the head and neck are anatomically classified considering: primary tumor size (T), regional nodal spread (N), and distant metastases (M). According to the Union for International Cancer Control (UICC), the classification goes from I to IV and stages III and IV define a more advanced disease (13). Most patients are diagnosed at an advanced stage of the disease (14), and around 40% of patients are already malnourished prior to diagnosis (15,16). One of the etiologic factors related to HNC is smoking, mainly in developing countries, whereas in developed countries the human papillomavirus (HPV) is a major contributor to the development of oropharynx neoplasia (11).

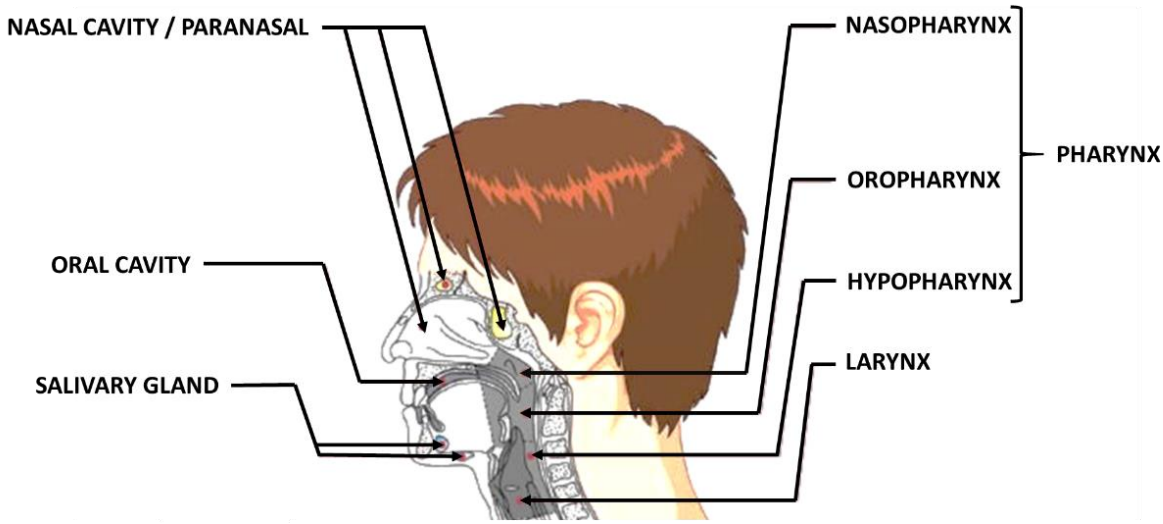

Figure 1: *Head and neck cancer subsites (adapted from* (10)*).*

Smoking is one of the most important risk factors for HNC once tobacco smoke contains chemicals that have cytotoxic and carcinogenic properties (12). The risk of developing HNC decreases about 30% 1-4 years after stop smoking, and about 60% after 10 to 15 years (17,18).

Alcohol is also a risk factor for the development of HNC, which seems to be due to the induction of cytochrome P4502E1, the formation of ROS and cell cycle deregulation (19). Studies show that alcohol consumption has more impact on oropharynx and hypopharyngeal cancer development than on the oral cavity or larynx cancer (20,21). In addition, stop drinking alcohol shows benefits as significant as stop smoking; however, only after 20 years or more of stop drinking the risk of developing HNC decreases approximately 40% (17). Moreover, the combination of these two HNC risk factors increases the probability of developing HNC (19).

HPV, mainly HPV type 16 and to a lesser extent type 18, is one of the causes of squamous cell carcinoma of the head and neck, especially in the oropharynx (22). It is estimated that over 50% of oropharyngeal squamous cell carcinomas of the head and neck are related to HPV infection (10). The development of cancer by this virus results from the integration of HPV DNA into the cellular genome leading to the accumulation of genetic and epigenetic alterations in genes involved in the control of proliferation (19). Another virus, the Epstein-Barr virus (EBV), mainly affects the nasopharynx (19). Indeed, EBV infection is detected in 100% of non-keratinizing nasopharyngeal

carcinomas (23). One of the hypotheses regarding the development of cancer in the nasopharynx by the EVB relies on the early genetic alterations promoted in the cells of the nasopharyngeal mucosa by latent EBV infection (19). Finally, Herpes Simplex virus (HSV) is an oral cancer promoter (24). The prevalence of HSV in malignant and potentially malignant lesions in the oral cavity is approximately 30%. HSV is involved in the activation of chromosomal mutations, gene amplification and overexpression of preexisting oncogenes within neoplastic tissue. The contribution of HSV to oral carcinoma progression is supported by the perseverance of the virus in the oral mucosa (25).

Other factors such as diet, oral hygiene, environmental carcinogens, family history, low body mass index, exposure to ultraviolet light and preexisting medical conditions might also predispose to HNC. The knowledge of the factors underlying HNC development at the time of diagnosis might help in therapeutic decision (26).

Treatment of patients with HNC may differ, and treatment options should be individualized for each patient (27,28). Treatments usually vary between surgery, radiation therapy or chemotherapy (27,29). Radiation therapy (RT) is used as the primary treatment or adjuvant therapy to surgery. However, patients with HNC who are treated with RT may have several adverse effects, including anorexia, oral mucositis, changes in taste, dry mouth, inflammation and swallowing problems, thus contributing to impairment of nutritional status. These effects depend on the treated area, volume, dose and the type and duration of RT, and on the patient's response (27). The use of surgery followed by radiation therapy is generally used in early-stage oropharyngeal and hypopharynx cancer (30). Isolated surgery is used as the first option when cancer is located in the oral cavity (28). Its use always depends on the location, and so radiotherapy and chemotherapy are a good alternative to surgery when it is difficult to reach places or when vital structures such as the carotid artery are involved (29). Besides these therapeutic options for the management of HNC, non-pharmacological approaches such as exercise training is recommended to improve quality of life of HNC patients. Regular moderate exercise, such as walking, has beneficial effects such as on pro-inflammatory cytokines regulation, with impact on morbidity and mortality (31).

1.2. Nutritional impairment in HNC patients

Patients with HNC are a nutritionally vulnerable group due to tumor- and treatment-related factors, and psychological and social elements that seriously compromise their nutritional status (32). The tumor itself can lead to dysphagia, usually seen in patients with locally advanced tumors (33). In addition, it can cause pain, increased inflammatory activity, and altered metabolic function (32). Malnutrition and nutritional deficits have been associated with a negative impact on HNC mortality, morbidity and quality of life (34). These are worrying facts since normally up to approximately 57% of the patients with HNC present malnutrition with a weight loss of 10% of the basal body mass. A high prevalence of weight loss has been reported even before treatment (32). Patients with HNC who present weight loss at the diagnosis stage may present a more aggressive disease and usually have a worse prognosis. For example, patients with early significant weight loss, i.e, more than 20% loss from baseline, are more likely to die from a treatment-related complication. Weight loss during treatment is common and, therefore it is not universally prognostic (35). This may be explained by the fact that some patients present weight loss caused by decreased caloric intake, while the cause in other patients is a manifestation of a syndrome of dysregulated catabolism and anabolism (36). In fact, studies show that weight loss occurring in patients with HNC both at diagnosis and during treatment is primarily a result of a loss of lean body mass, and cannot be reverted by parental nutrition (37,38). In addition, several studies have demonstrated the importance not only of weight but of changes in body composition throughout cancer treatment (39,40). Therefore, weight loss in HNC patients is usually associated with a syndrome called cachexia.

1.3. HNC cachexia-related muscle wasting: circulating mediators and signaling pathways

Cachexia is “a multi-factorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment” (41). Cachexia is an underexplored syndrome in the set of HNC (42), being associated with increased rates of mortality, morbidity and impaired quality of life in patients with HNSCC (43). Cachexia is characterized by decreased appetite, weight loss, metabolic alterations and inflammatory state (43). In addition, patients present decreased physical

performance, which impairs their quality of life and the clinical outcome of disease treatment (44). More than half of cancer patients suffer from cachexia at the time of death. Initially, it was estimated that cachexia contributes to cancer death in approximately 20% (34); however, there is no consensus in the literature (35,36). For instance, percentages of 20% or higher were reported in HNC patients (32). Cachexia is hardly diagnosed in early stages, i.e., in pre-cachexia stages, which are characterized by involuntary weight loss lower than 5% up to 6 months, and/or by metabolic changes, such as increased proteolysis, increased lipolysis, increased resting energy expenditure and insulin resistance (31,32). However, cachexia is already established when there is an unintentional weight loss of at least 5% over 3 to 6 months or weight loss > 2% over 6 months in individuals with a body mass index < 20 kg/m^2 in absence of simple starvation or the presence of sarcopenia (37,38). The identification of patients in the initial stage of cachexia is essential for early interventions (45). Thus, it is very important to understand the molecular pathways and mediators involved in the pathogenesis of cachexia and to know how to identify this syndrome. Mediators of systemic inflammation have been the most recognized cachexia markers, especially acute phase response proteins (45). Some cytokines such as interleukin (IL) -1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α), and Interferon-gama (IFN-γ) have already been linked to the cachexia pathogenesis in some cancers such as esophagus, pancreas, lung, and prostate. In addition, cachexia may result from the deregulation of neuropeptides, such as α-melanocyte stimulating hormone, neuropeptide Y, melanocortin, and corticotrophin-releasing factor (46). Richey *et al.* reported an association of IL-6 and C-reactive protein (CRP) with cachexia in patients with HNSCC (46). The cytokines IL-1β, IL-6, IFN-γ and TNF-α have also been associated with muscle wasting (47). This cancer-related inflammation has been related with the communication between the host and tumor cells. This interaction usually results in systemic alterations, immune suppression and evasion, and malignant progression (48). Indeed, the increase in CRP is associated with tumor proliferation, increased metastases to lymphatic, and worse prognosis (49). In contrast, in a study conducted by Wittenaar *et al.* no marked inflammatory activity was observed in patients with cachexia, compared with the ones without cachexia (42). Indeed, it has been noticed a variability in inflammatory activity in patients with advanced cancer stage across studies in literature (46,50).

Cachexia seems to result from the activation of some pathological muscle wasting mechanisms, with inflammation and oxidative stress being the triggering players of the imbalance between protein synthesis and breakdown (51,52). The best defined anabolic pathway in skeletal muscle involves the activation of the serine/threonine kinase Akt that results in the activation of the mammalian target of rapamycin (mTOR) (52). However, the activity of this pathway is reduced, if not abolished, in catabolic diseases, such as cancer cachexia (53). In overall, the molecular pathways associated to muscle catabolism are: (a) the ubiquitin-proteasome pathway and caspases under transcriptional control of the transcription factors forkhead O (Fox-O) and nuclear factor (NF) -kB (54); (b) the myostatin (Mstn) pathway (55), which acts locally as a negative regulator of the Akt/mTOR pathway and by the decreasing the number of satellite cells; (c) muscle apoptosis (56); (d) autophagy/lysosomal activity (57). The molecular triggers and the signaling pathways under their control in skeletal muscle are critically analyzed in the following subsections.

1.3.1. Interleukin-6

IL-6 is a glycoprotein, mainly produced as an acute-phase protein by the liver and it is related to the development of cachexia. In addition, some tumors secrete IL-6; however, this cytokine is predominantly secreted by immune cells. It is involved in the amplification of inflammatory cascades in the immune response. Elevated levels of this protein are detected in patients who have cancer cachexia (31,47,58). High levels of IL-6 have been reported in HNSCC (59–62). Moreover, IL-6 concentration seems to increase as tumor stage progresses, with significant differences detected between stage IV and previous stages of cancer (62).

The IL-6 signaling mechanism begins by its binding to an 80 kDa type 1 cytokine alpha receptor subunit designated IL-6R or CD126 and to 130 KDa, gp130 forming an IL-6/ IL-6R/gp130. From this complex different signaling pathways can be followed, such as the JAK/STAT3 pathway or the PI3K/Akt, Ras/MAPK and Wnt signaling pathways (63). The activation of JAK/STAT3 seems to explain the loss of muscle mass in cachexia (64,65).

Muscle atrophy in cancer cachexia besides being caused by increased protein degradation is also caused by decreased protein synthesis. Inhibition or down-regulation of the PI3K/Akt/mTOR pathway is linked to decreased protein synthesis. IL-6 in

conjunction with serum amyloid-A (SSA), increases SOCS3 expression in muscle. This molecular player leads to the down-regulation of IRS-1. Since the PI3K/Akt signaling pathway requires the initial activation of IRS-1, this pathway is inhibited by SOCS3 and so protein synthesis decreases (64). The role of IL-6 on proteolysis was corroborated by studies that injected IL-6 into rodents observed muscle protein breakdown, while when IL-6 receptor antibody was administered muscle atrophy was prevented (66–68).

The IL-6/gp130/JAK/STAT3 pathway has been associated with cachexia. IL-6 initiates STAT3 activation by binding to gp130 and activating JAKs that phosphorylates STAT3. In addition to IL-6, STAT3 can be activated from leptin through the homologous receptor to gp130. STAT3 can also be activated by tyrosine kinase receptors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), among others (65). Following the phosphorylation of STAT3, spontaneous dimerization of this transcription factor occurs. This pathway ends with the phospho-STAT3 being translocated to the nucleus where the dimers bind to the consensus sequences on the promoter regions of target genes with the resultant cascade of gene (69) (Figure 2). To date, the mediators involved in the activation of STAT3, the IL-6 family are the best characterized in cancer cachexia, especially the IL-6 itself. It has already been shown that IL-6 is enough to induce weight loss in rats (70). In addition, the STAT3 phosphorylation increases with the severity of cachexia and serum concentrations of IL-6 (71). On the other hand, in another study, mice with muscle-specific deletion of gp130 and Lewis lung carcinoma cachexia had reduced muscle loss and reduced pSTAT3 (72). The results obtained by this study are like those of a study where cachexia associated to intestinal cancer was treated with IL6R neutralizing antibodies (73).

In HNSCC, the activation of STAT3 seems a common event; however, STAT3 mutations with gain-of-function were not observed, neither activating mutations in the EGFR or JAK receptors (69). Therefore, the positive regulation of STATs seems to be done through the activation of cytokine or growth factors receptors or by the negative regulation of protein tyrosine phosphatase receptors (PTPR). Thus, hyperactivation of STAT3 may be a consequence of upregulation of upstream signaling players, such as IL-6 or of decreased activity of upstream negative effectors (74,75). So, STAT3 signaling might be a target for the development of HNSCC therapy. The interruption of this pathway could be made by: (i) inhibiting upstream receptors and thereby reducing phosphorylation; (ii) inhibiting the pSTAT3 SH2 domain, blocking dimerization; (iii)

inhibiting STAT3-DNA binding, thus preventing target gene transcription; and (iv) by inhibiting STAT3 transcription, to modulate total expression of STAT3 (69).

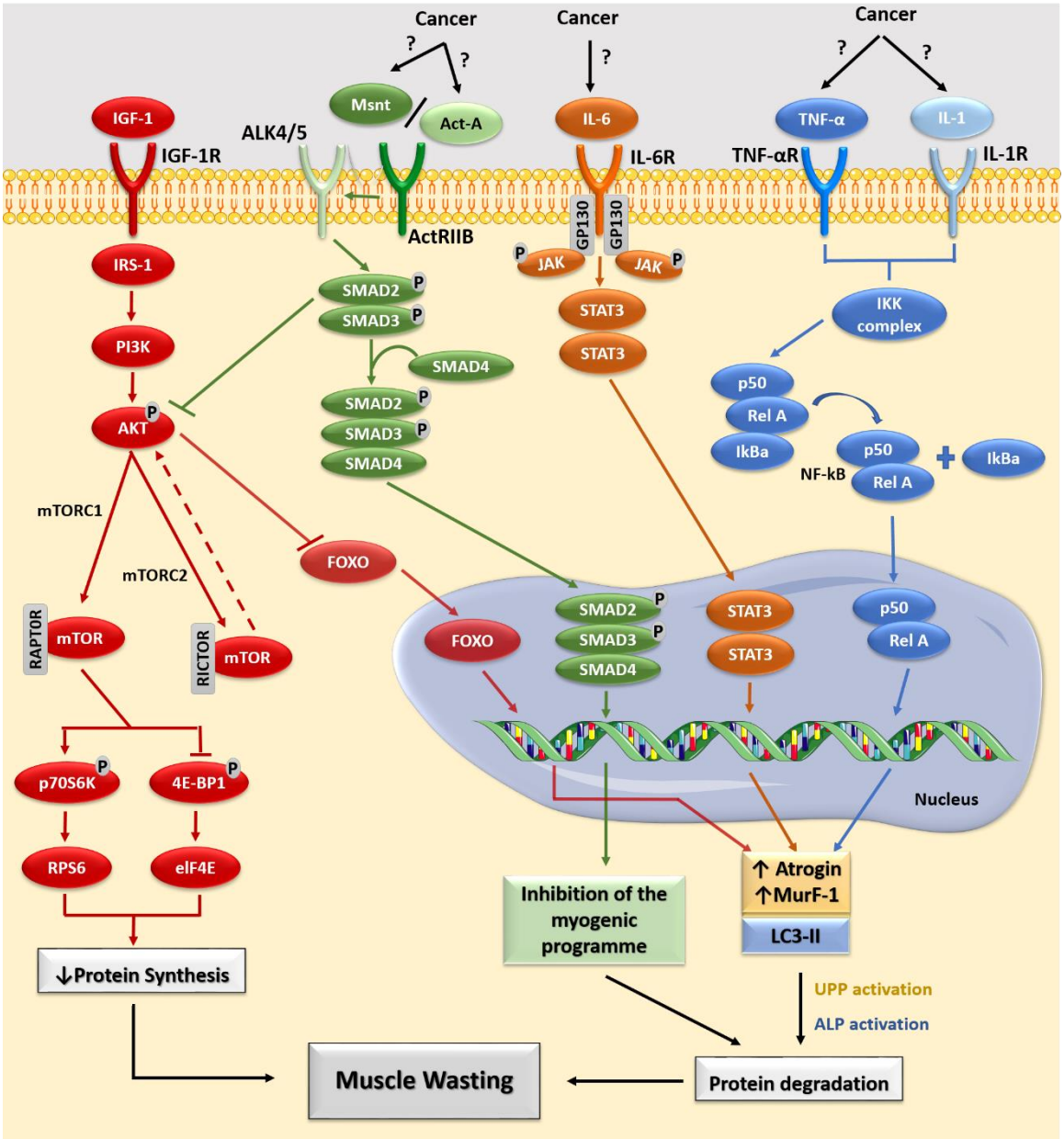

Figure 2. *Signalling pathways modulated by cancer in skeletal muscle that results in muscle wasting. In IGF-1 / Akt / mTOR signaling pathway, occurs phosphorylation of the IRS insulin receptor after binding of IGF-1 to IGF1R results in the activation of PI3K / Akt signaling, which upon activation of mTOR culminates in the protein synthesis. The Myostatin and Activin A signaling depends initially on the binding of one of the mediators (Activin or Myostatin) to the common receptor, ActRIIB on the muscle membrane, resulting in its dimerization and subsequent recruitment and activation of type 1 receptors. Next, SMAD2/3 phosphorylation takes place, on the part of type 1 receptors, that recruits SMAD4. The complex formed is translocated to the nucleus leading to transcriptional changes that result in protein degradation. On the other hand, SMAD2/3 phosphorylation suppresses the FoxO3 phosphorylation that is translocated to the*

nucleus and contributes to protein degradation through the activation of the MuRF-1 and Atrogin-1 genes and autophagy. In addition, SMAD2/3 phosphorylation reduces the activity of Akt without protein synthesis. In case of JAK/STAT3 signaling pathway, this is active after IL-6 binding to the receptor following induction to gp130 homodimerization. After phosphorylation of STAT3, a dimer is formed which is translocated to the nucleus which results in the increase of atrogin and MuRF1 culminating in protein degradation. In addition, TNF-α and IL-1 also activate ubiquitination from activation of the IKK complex. The complex phosphorylates the IkBa proteins and the NF-kB is translocated to the nucleus, where ubiquitination and protein degradation are activated, resulting in the loss of skeletal muscle mass. Figure produced using the Servier Medical Art.

1.3.2. Tumor necrosis factor-α

TNF-α plays central roles in inflammation, apoptosis, and development of the immune system (76). It is released by activated macrophages and other cells such as CD4+, neutrophils, mast cells, eosinophils, and tumor cells (77). The mechanism of action remains largely puzzling, it is only known that TNF-α can stimulate catabolism via indirect mechanisms (78). These mechanisms may involve alteration of circulating levels of hormones that regulate muscle growth and affect tissue sensitivity to such factors, stimulation of catabolic cytokine production, induction of anorexia (weight loss, loss of appetite, increased thermogenesis) and alterations of carbohydrates, proteins and lipids metabolism (78,79). Llovera *et al*. reported an increase of skeletal muscle protein degradation in rats after TNF-α administration (80). The same effect was observed by others in genetically modified mice with no TNF-α receptor protein type I and in wild-type mice transplanted with Lewis lung carcinoma (81).

TNF-α is a cytokine that contributes to cachexia by increasing corticotrophin releasing-hormone (CRH) levels and decrease food intake (77). It also participates in the recruitment of inflammatory cells that contribute to the degradation of muscle tissue proteins (82). TNF-α also promotes muscle mass loss by inducing apoptosis of muscle fibers (83). In addition, high levels of TNF- α have been related to the short survival time of patients with HNSCC (84). This evidence contributes to the hypothesis that higher levels of TNF-α are characteristic of patients with HNC cachexia (85).

TNF-α stimulates the degradation of myofibrillar proteins, which was more notorious in the *soleus* muscle of rats after acute treatment with recombinant TNF-α. The same was not observed in the *extensor digitorum longus* muscle (86). It has already been demonstrated that protein redistribution and marked depletion of muscle protein occurs

only after a long treatment with recombinant TNF-α (87). The role of TNF-α in protein degradation occurs mainly through the catabolic nuclear factor kappa B (NF-kB) signaling (88). NF-kB plays an important role in regulating the ubiquitin-proteasome system (UPS), which is the principal mechanism of protein degradation in muscle cells. Besides being regulated by NF-κB signaling, the UPS is further regulated by O-protein FoxO, p38, mitogen-activated protein kinase (MAPK) and STAT3 (89). TNF-α promotes NF-kB activation in the skeletal muscle through the activation of the type 1 TNF- α sarcolemmal receptor (TNFR1) (90). Consequently, protein kinase C (PKC) is activated and results in the rapid conjugation of ubiquitin to muscle proteins (85,90,91). All these processes lead to the proteasomal degradation of I-kBa, releasing NF-kB subunits from its inhibitory effect. Then, NF-kB dimers translocate to the nucleus (85), where regulates the expression of genes from the UPS. The proteins selected to be broken down will be ubiquitinated, i.e., are marked by covalent linkage of a chain of ubiquitin molecules to an internal lysine on the protein. Later, ubiquitinated proteins are recognized and degraded by the proteasome in smaller peptides. This process is regulated by three types of enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3). E1 is the enzyme that initiates the pathway of the ubiquitin-proteasome, since the first step is the activation of the ubiquitin monomers and their linked to E1, then they are transferred to E2, and from there they can interact with an E3 and transferred to the substrate protein (64). E3 is the key enzyme in the UPS, in fact, its function is to bind the activated ubiquitin to the lysine residues on the protein substrate and confers specificity to the system. The E3 ubiquitin ligases responsible for the degradation of the skeletal muscle protein are atrogin-1, also referred to as MAFbx/Atrogin, and muscle RING finger protein 1 (MuRF1). The targeted deletion of atrogin-1 and/or MuRF1 showed reduction of muscle atrophy, confirming its role in the skeletal muscle-wasting (54). In the end, after interaction with E3 and transfer to the substrate protein the proteins that were ubiquitin-marked are degraded by the 26S proteasome complex (20S core catalytic complex and one or two 19S regulatory complexes). The 19S regulatory complex is responsible for substrate recognition (64). Cai *et al*. demonstrated that NF-κB activation resulted in muscle wasting in transgenic skeletal muscle selective IKKβ mice. E3 ligase MuRF1 was also shown to be increased in these mice (92). Thus, when there is an increase of TNF-α the UPS is stimulated in excess, culminating in the loss of muscle mass (Figure 2). So, TNF-α can promote protein

degradation, with its mediators being NF-kB. Despite its role in promoting muscle wasting, many patients with HNC-related cachexia showed unchanged circulating levels of TNF-α, suggesting that this cytokine is a poor prognostic marker of cachexia. Moreover, TNF-α inhibition did not result in the stop or in the reverse of cancer cachexia (93).

1.3.3. Interleukin-1

IL-1 is a pleiotropic pro-inflammatory cytokine involved in tumor growth and metastasis. Within the IL-1 family, there are three members that bind to the same receptor, IL-1α and IL-1β, two active members and the third member is an antagonist, IL-1Ra (94). The IL-1 pathway is involved in inflammatory responses, its role is to regulate the expression of various inflammatory genes in immune cells. The IL-1 pathway is activated when one of the agonists, IL-1α or IL-1β, binds to the IL-1 receptor (IL-1R1). This IL-1 signaling pathway has been associated with tumor growth, angiogenesis, metastasis, and cancer cachexia. This association occurs due to the role of this pathway in the expression of various proinflammatory cytokines, including IL-1, IL-1β, IL-6, IL-8, involved in tumor survival, and is involved in the infiltration of several immune cells in the tumor, which increases the inflammatory and pro-survival microenvironment of the tumor (95). In addition to TNF-α, IL-1 is an activator of NF-κB. There is evidence of aberrant constitutive activation of NF-kB in the HNSCC tumor cells. By regulating the activation of NF-kB and also by triggering the cascade expression of chemokines/cytokines detected in SCC, IL-1 initiates proinflammatory responses. In addition, IL-1 can function as an autocrine factor, i.e., it stimulates tumor fibroblasts to produce IL-6, IL-8, GM-CSF, VEGF, and a paracrine factor to stimulate HGF production by stromal fibroblasts (96). Elevated levels of IL-1 produced by tumor cells have been associated with poor prognosis (97) and with increased risk of distant metastasis (98).

IL-1 has also been associated with HNC cachexia (47,99), which seems to involve the activation of NF-kB signaling in skeletal muscle (100). IL-1 concentrations increase in cachexia and their effects are like TNF-α relative to the NF-kB pathway (93). That is, IL-1 binds to its IL-1R receptor, activating the IKK complex, which phosphorylates the IkB (NF-κB inhibitors) proteins, marking them for poly-ubiquitination and proteosomal degradation releasing the NF-κB which activates MuRF1 and atrogin-1, two ligases that promote protein degradation (Figure 2) (101). Moreover, IL-1 induces anorexia in

cachectic patients, as it induces an increase in tryptophan concentrations, which leads to increased levels of serotonin, since it is its precursor. Serotonin may play a role in the development of cancer cachexia since it seems to regulate the appetite at the hypothalamic level (102). Plasma levels of IL-1β have been shown to better reflect the characteristics of cachexia (weight loss and sarcopenia) than IL-6. They have been associated with weight loss, sarcopenia, asthenia, and loss of appetite (103).

1.3.4. C-reactive protein

CRP is an acute phase protein and functions as a marker of inflammation, not only acute but also chronic (104,105). Its synthesis occurs in hepatocytes and can be regulated by proinflammatory cytokines, including IL-1, IL-6, and TNF-α (104). In HNC patients, an association between elevated CRP, TNF-α, and IL-6 has been demonstrated (106–108). Therefore, when there is an abundance of proinflammatory cytokines in the tumor microenvironment, this can potentiate angiogenesis, favoring neoplastic growth. Patients with malignant tumors normally have high serum CRP content, which highlights a close connection between inflammation and malignancy (105). Patients with oral squamous cell carcinoma and elevated serum CRP levels before treatment presented a worse prognosis, with nearly all patients surviving at most 5 years, whereas patients with normal CRP levels had survived after 5 years of surgical resection (109). In addition to promoting neoplastic growth, elevated CRP has been positively correlated with weight loss and cancer cachexia (110,111). Its association to muscle loss seems to be explained by the contribution of skeletal muscle in supporting other tissues with free amino acids to sustain their protein synthesis, namely the synthesis of acute phase proteins in the liver, including CRP. An example of such an amino acid is glutamine, a non-essential amino acid, which is released by skeletal muscle in order to provide energetic substrate and precursor to nucleic acid synthesis for rapidly dividing cells, the immune system, and the intestinal mucosa. Glutamine is also a primary source of nitrogen that supports tumor cells proliferations once supports protein and DNA synthesis, being also a source of energy (112,113). Alanine is other example of an amino acid exported in large quantities from skeletal muscle and used for the synthesis of proteins in other tissues. This amino acid also supports liver gluconeogenesis, being an energetic substrate of tumor cells. In an inflammatory situation, the muscle becomes inefficient to provide enough amino acids to

the entire body, eventually resulting in catabolism (93,114). So, the large amounts of glutamine and, indirectly, alanine, that the tumor consumes along with the use of these amino acids for the synthesis of the acute phase proteins have a synergistic effect on the loss of muscle mass.

1.3.5. Myostatin and Activin-A

Activin-A (ActA) is a homodimer of β-Activin chains, belonging to the TGF-β superfamily. The concentrations of ActA increase in circulation with acute inflammation, acute respiratory failure, renal failure and in some cancers, especially in cancers with bone metastases (115). Activin may be present in the circulation in bioactive free form or bound to Follistatin (116). There is evidence that inhibition of ActA results in reversal of cachexia and thereby increases survival in animal models identified with cancer cachexia (117). In HNC, high expression of ActA has been associated with a more aggressive tumor, since the high expression of ActA is correlated with tumor differentiation, metastasis, survival, and recurrence (116).

Mstn is also a member of the TGF-β superfamily and is involved in the negative regulation of skeletal muscle growth and differentiation. Mstn is secreted by murine and human neoplasms and so, this cytokine was proposed to be a novel tumoral factor that stimulates skeletal muscle wasting during cachexia (118). High circulating levels of Mstn were reported by several authors in muscle wasting conditions (119–122). However, contradictory results were described. Loumaye *et al*. reported lower plasma concentrations of Mstn in cachectic patients when compared with non-cachectic patients (115). So, the role of Mstn on HNC cachexia is still elusive.

ActA is believed to replicate the biological action of Mstn on the skeletal muscle since they share the same receptor (115). The mechanism of action of Mstn and ActA in skeletal muscle is shown in Figure 2. The released Mstn or ActA binds to the receptor B of the membrane activin type II (ActRIIB). After binding of Mstn or ActA, ActRIIB undergoes dimerization, with recruitment to the membrane of type I receptor (ActA: ALK4 or 7; Mstn: ALK5 or 7). The already activated type I receptor phosphorylates SMAD2/3 which forms a complex with SMAD 4 and this complex will regulate the transcriptional responses that result in the loss of muscle mass. In addition, Mstn and ActA succeed in suppressing the activity of Akt, therefore the activity of mTOR resulting

in decreased protein synthesis. This Akt inhibition has another consequence since Akt can phosphorylate and inactivate the O-proteins of the graph box (FOXOs: FoxO1, FoxO3, and FoxO4) and these are exported from the nucleus to the cytoplasm. With Akt activity inhibited FoxO3 is not phosphorylated, ie it is translocated to the nucleus. In this way, they will contribute to the loss of muscle mass since they are involved in the transcriptional regulation of autophagy that promotes the ubiquitination and degradation of proteins (101).

1.4. Strategies for the management of HNC muscle wasting targeting the molecular mediators involved

Cancer cachexia is a highly incident syndrome in patients with HNC and of great clinical importance. However, there are no broadly adopted guidelines to standardize its management. To aggravate the situation, there is a lack of effective pharmacological interventions for the treatment of this syndrome. Currently, the approaches used are mainly non-pharmacological, such as nutritional education, support, and exercise. Some pharmacological agents have been recommended as corticosteroids and progesterone analogs; however, the positive effects are mainly at the anorectic level, and their clinical use is limited due to its side effects. In this way, efforts have been made in the attempt to develop novel strategies for the management of this syndrome. Some agents have already been studied, including ghrelin analogs and anti-cytokines, the latter being previously seen as possible mediators in HNC cachexia (123).

Cytokine inhibitors or anti-cytokines may be potential effective agents in the treatment of HNC cachexia. Inflammation is one of the main promoters of cachexia and these agents may be potentiating modulators of inflammatory catabolic conditions (124). Pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6 are the main interveners under pathophysiological conditions that leads to chronic inflammation and loss of skeletal muscle. Their presence in large concentrations in the blood of patients with cachexia makes them potential targets of treatment in patients with HNC cachexia. That is, antibodies against these cytokines may have a positive effect on the regulation of skeletal muscle loss in patients with HNC cachexia (125). Some examples of the anti-cytokines drugs are thalidomide, clazakizumab, MABp1, IP-1510, among others (123).

Thalidomide is a derivative of glutamic acid, this agent has already been shown to have anti-inflammatory, immunomodulatory, anti-angiogenic, sedative and anti-emetic effects. Its action has been extensively studied, since this agent appears to involve the suppression of several cytokines, including TNF-α and IL-6, but also angiogenesis mediators, such as VEGF and FGF, inhibition of NF-κB and downregulation of COX-2 (123,125). Besides, thalidomide was reported to reduce serum levels of CRP in patients with cachexia (125). In contrast with some anti-cytokines agents, thalidomide has been shown to be well tolerated (126,127). Even, the effectiveness of thalidomide in the treatment of cachexia is questionable, and further studies are needed. Putative anti-TNF strategies for the treatment of cancer cachexia, such as pentoxifylline, etanercept, infliximab, and melatonin, have been studied and suggested (123). Administration of anti-murine TNF IgG has been shown to down-regulate the levels of circulating TNF in rats (128). Regarding IL-6, it has been observed that the IL-6 inhibition contributes to the non-development of cachectic features. Then, it became an interest to target this pro-inflammatory factor in the clinical setting. The humanized monoclonal antibody clazakizumab (ALD518), the only specific anti-IL-6 agent, has been evaluated in clinical trials in the treatment of cancer cachexia (123). ALD518 is well tolerated, with minimal adverse effects (101). And, therefore, it could be a potential agent to introduce in the management of HNC cachexia; however, these putative beneficial effects must be confirmed. IP-1510 is a synthetic peptide IL-1 receptor antagonist. However, limited efficacy and toxicity for cancer cachexia has been reported (129). MABp1 is more specific because it is a natural IgG1k human monoclonal antibody targeting IL-α, since it is an early mediator of the inflammatory response (123). MABp1 has been shown to be well tolerated, effective on body composition and seems to have antitumor effects. Despite these promising results in patients with cancer cachexia, this antibody has been shown to cause proteinuria (130).

As noted above, Mstn and ActA inhibit muscle growth and promote muscle protein loss. And, therefore, they can also be two therapeutic targets for the treatment of cancer cachexia. And in this way, two human monoclonal antibodies against myostatin, bimagrumab (BY338), and trevogrumab (REGN1033), have been studied. The first showed potential to increase the differentiation of primary human skeletal myoblasts and to increase the skeletal muscle mass in mice since it can block the ActRIIB receptors (125). The BY338, in addition to binding to the ActRIIB receptor, has more affinity than

the natural ligands Mstn and ActA (131). Despite its success in the animal model, there is a lack of studies in humans to assess its effectiveness and absence of adverse effects. REGN1033 has high affinity and specificity for myostatin. It has been shown to increase muscle strength in young and old mice, and to prevent muscle mass loss, accelerate muscle mass recovery and promote better physical performance in combination with exercise in mice (132). Again, its disadvantage is that all these characteristics were observed only in animal models.

Other agents that have also been studied as a strategy for the management of cancer cachexia were ghrelin analogs (123). The clinical use of ghrelin becomes even more difficult because ghrelin has a short half-life and parenteral dosage form. In this way, other options have been investigated. A ghrelin receptor agonist called anamorelin has advantages over ghrelin as it can be administered orally and has shown positive effects on appetite and lean body mass (124). However, despite increasing muscle mass this ghrelin agonist does not increase muscle strength. This result is not surprising, since ghrelin increases muscle mass by increasing growth hormone, which not modulates muscle strength. Despite this, its use in the treatment of cancer cachexia should not be totally ruled out once it improves appetite and, thus, the anorexia usually seen in patients with HNC cachexia (133). Therefore, the use of anamorelin for the treatment of cancer cachexia should better explored to assess efficacy and long-term safety (124).

2. AIMS

The general objective of this dissertation was to characterize the muscle wasting phenotype of HNC patients and identify putative biomarkers to improve the early diagnosis and management of muscle wasting in this group of patients. To accomplish this aim, serum and urine samples were collected from patients with HNC, with and without body weight loss, for proteome characterization using target and non-target strategies. Immunoblotting and mass spectrometry-based approaches were used for the identification of the biological processes involved in HNC-related body wasting and the underlying proteins.

3. MATERIAL AND METHODS

3.1. Patients selection

Thirty male patients (aged 43–77 years) diagnosed with head and neck cancer were enrolled in the present study, which was conducted between September 2018 and June 2019. Study protocol was approved by the Ethics Committee from the *Instituto Português de Oncologia do Porto*. The nature and purpose of the study was explained to participants before written informed consent was obtained. The eligibility criteria included patients: i) no obese and no diabetic; ii) no prior history of cancer; iii) with spinocellular tumors; iv) not submitted to prior cancer treatment; v) able to do unrestricted physical activity. Clinicopathological data was obtained from patients' clinical records. Among the information gathered by physicians from the *Instituto Português de Oncologia do Porto* was age, smoking and drinking habits, medication and tumors' stage, nutritional information regarding body mass index (BMI) and Malnutrition Universal Screening Tool (MUST), shown in Table 2. In addition, biochemical data from serum analysis (C-reactive protein (CRP), albumin, glucose, urea, cholesterol, and triglycerides) were provided. The reference values are shown in Supplemental Table S1. HNC tumours were histologically classified according morphologic characteristics that allow the identification of histogenesis of the tumor as epidermoid/squamous (134). Blood and urine samples were collected from the enrolled patients and prepared for targeted and non-targeted proteomic approaches, as described in the Figure 3.

Table 2. *Clinical and pathological data of the patients included in the study.*

Gender, n (%)		
Male		30 (100.00)
Smoking habits, n (%)		
None		0 (0)
Previously smoked		7 (23.33)
Smoker		23 (76.67)
Alcoholic habits, n (%)		
None		6 (23.33)
Light		2 (6.66)
Moderate		4 (13.33)
Marked		16 (53.33)
Chronic		1 (3.33)
BMI, n (%)		
Underweight (<18.5 Kg/m^2)		11 (36.67)
Healthy weight (18.5 – 25 Kg/ m^2)		17 (56.67)
Overweight (>25Kg/ m^2)		2 (6.67)
Location of the tumor, n (%)		
Tongue		8 (28.57)
Pharynx		10 (35.71)
Larynx		3 (10.71)
Laryngeal Pharynx		6 (21.43)
Vocal cord		1 (3.57)
Tumor staging, n (%)		
stage T	0	0
	1	3 (10.34)
	2	2 (6.90)
	3	9 (31.03)
	4	15 (51.72)
stage N	0	8 (27.59)
	1	1 (3.45)
	2	16 (55.17)
	3	4 (13.79)
MUST score, n (%)		
0		9 (34.62)
1		4 (15.38)
2		7 (26.92)
3		3 (11.54)
4		3 (11.54)

There was no information for one patient about tumor location and other patient had 3 tumors (nasopharynx, pyriform sinus, and lung). The latter patient was further removed from the information on tumor staging. For the MUST score, information was missing for 4 patients. The n represents the number of individuals considered in each parameter.

Patients were initially assigned to one of two groups according to the percentage of body weight loss reported in the last 6 months. Patients that reported a weight loss higher than 5% were included in the head and neck cancer + cancer cachexia (HNC+Cachexia; n=20) group whereas patients with no weight loss or weight loss lower than 5% in 6 months were included in the head and neck cancer with no cachexia (HNC; n=10) group. After the analysis of experimental data and considering previous findings on the evaluation of body wasting (135), patients were redistributed based on nutrition risk index (NRI), described below. According to this distribution, 16 patients were assigned to HNC group and 14 patients to HNC+Cachexia group.

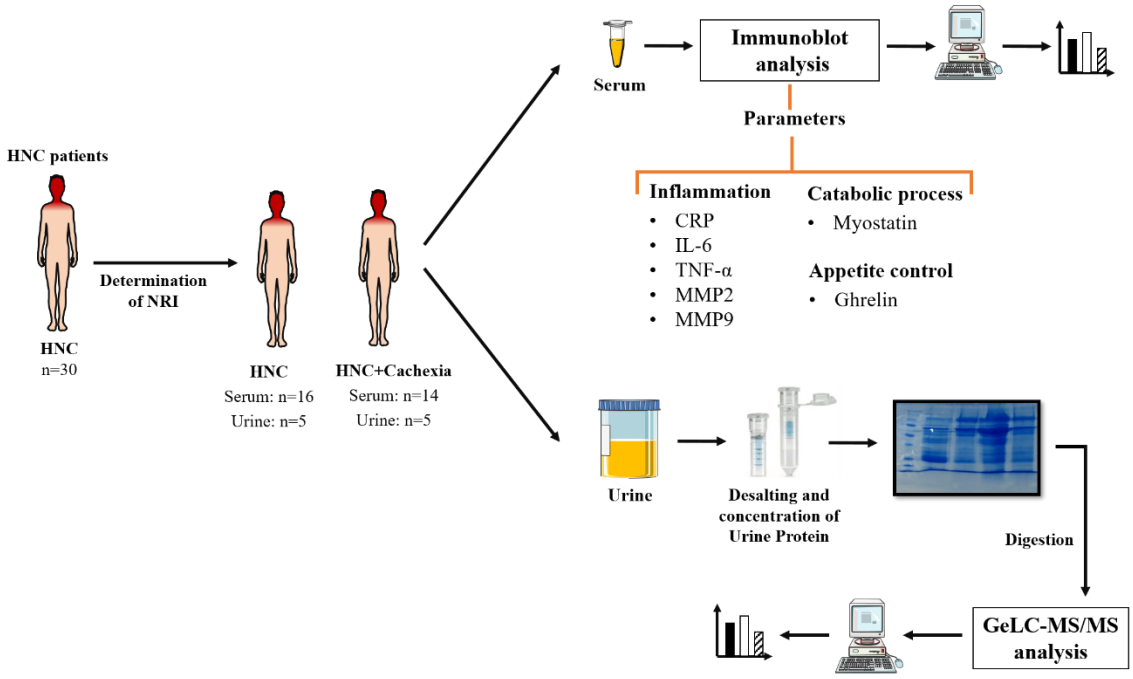

Figure 3. *Schematic representation of the experimental procedure. Serum and urine samples were collected from patients. Serum was used for the immunoblot analysis of protein targets whereas urine proteome was characterized by GeLC-MS/MS.*

Legend: CRP: C-reactive protein; HNC: head-and-neck cancer; IL: interleukin; TNF-α: tumor necrosis factor alpha; MMP: metalloproteinase; MS: mass spectrometry. Figure produced using the Servier Medical Art.

3.2. Nutritional risk assessment

Nutritional risk was assessed through the application of the Nutritional Risk Index (NRI) tool developed by the Veteran Affair Total Parenteral Cooperative Study Group (136). This is a tool with high sensitivity and specificity (137,138). The NRI is determined using the values of serum albumin, and of the actual and usual percentage weight, based on the following equation:

$$NRI = 1.519\ x\ Alb\ serum\ \left(\frac{g}{dL}\right) + 0.417\ x\ \left(\frac{actual\ weight}{usual\ weight}\ x\ 100\right).$$

The following criteria were used for the analysis of results: above 100 indicate satisfactory nutritional status; between 97.5 and 100, mild risk of malnutrition; from 83.5 to 97.5, moderate risk; and below 83.5, severe risk.

Patients with NRI equivalent to a satisfactory nutritional status or mild risk were considered without cachexia and, thus included in the HNC group. Patients with moderate or severe risk were considered with cachexia and included in the HNC+Cachexia group.

3.3. Immunoblot analysis of Serum samples

Blood samples were collected in the morning at IPO-Porto. No fasting was required to patients. Blood samples were allowed to clot for one hour and then centrifuged at 4000g for 10 minutes. The supernatant was collected and stored at 20ºC until the analysis of some molecular markers by immunoblot analysis performed according to Caseiro et al. (139) with slight modifications.

Samples were diluted (1:50) in Tris-buffered saline (TBS; 10 mM Tris, pH 8.0, 0.15 M NaCl). Then 50 µL of each of these samples were blotted onto a nitrocellulose membrane (Amersham™ Protran™, GE Healthcare, Germany, 0.45 µm porosity) under vacuum, followed by Ponceau S staining for protein loading control. Nonspecific binding was blocked with 5% (w/v) nonfat dry milk in TBS with 0.05% of Tween 20 (TBS-T), during 1 hour at room temperature with agitation. Next, the membranes were incubated with the corresponding primary antibody, diluted 1:1000 in 5 % (w/v) nonfat dry milk in TBS-T for 1 hour at room temperature and with agitation. The primary antibodies used were: rabbit polyclonal anti-ghrelin (ab64325), rabbit polyclonal anti-IL-6 (ab6672), rabbit polyclonal anti-myostatin (ab98337), rabbit polyclonal anti-C reactive protein (CRP; ab32412), mouse monoclonal anti-TNF-α (ab1793), rabbit polyclonal anti-

metalloproteinase (MMP) 2 (ab37150) and rabbit polyclonal anti-MMP9 (ab38898) from Abcam® (Cambridge, UK). Then, the membranes were washed 3 times (10 minutes each) with TBS-T and incubated with the appropriate secondary horseradish peroxidase-conjugated antibody (anti-mouse or anti-rabbit, NA931 or NA934, respectively; GE Healthcare, UK), diluted 1:1000 in 5% (w/v) nonfat dry milk in TBS-T for 1 hour at room temperature with agitation. Membranes were then washed 3 times with TBS-T (10 minutes each). Immunoreactive bands were detected with ECL reagents (WesternBright™ ECL, advansta, CA, USA) using the ChemiDoc Imaging System v.2.3.0.07 (Bio-Rad®, CA, USA) and the images obtained were analyzed with Image Lab software (Bio-Rad®, CA, USA, version 6.0.0,). The optical densities obtained were expressed in arbitrary units.

3.4. Proteomic characterization of urine samples

Patients provided one sample of urine collected in the morning, during their interview at IPO-Porto, collected on the same day of blood samples. Urine was centrifuged for 10 min, at 4000×g, 10-12°C. Then, 800 µL of the supernatant was passed through 10-kDa filters (Vivaspin, Sartorius), as previously reported (140). The retentate was resuspended in resuspension buffer (10% SDS and Tris buffer 0.5 M, pH 6.8). The protein content in the concentrated and desalted urine samples was assayed with the commercial kit RC DC™ Protein Assay (Bio-Rad®, Hercules, CA, USA), according to the manufacture's recommendations and using bovine serum albumin (BSA) as protein standard. A calibration curve with standard solutions of BSA at concentrations between 0.312 and 10 mg/mL was made. The absorbance was measured at 750 nm in a microplate reader (Multiskan GO, Thermo Fischer Scientific®, Northumberland, UK).

3.4.1. GeLC-MS/MS analysis

Equal amounts of protein (30 µg) from each concentrated urine sample were dissolved (1:2) in loading buffer (0.5 M Tris-HCl pH 6.8, 4% (w/v) SDS, 15% (v/v) glycerol, 1 mg/mL bromophenol blue and 20% (v/v) β-mercaptoethanol) and incubated at 100°C for 5 minutes. Subsequently, the samples were loaded in a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) prepared according to Laemmli (141). The gels were run for 45 minutes at 180 V in running buffer (250 mM

glycine, 25 mM Tris, pH 8.6 and 0.1% (w/v) SDS). Then, the gels were incubated in fixation solution (40% (v/v) methanol and 10% (v/v) glacial acetic acid) for 30 minutes and, after being washed with water, gels were stained with Colloidal Coomassie Blue G250 and destained with 25% (v/v) methanol until an optimal contrast was achieved. The gels were then scanned in the ChemiDoc Imaging System v.2.3.0.07 (Bio-Rad®, Hercules, CA, USA). Then, protein bands were cut from the gels (each sample's gel lane was divided into 5 fractions) and placed into eppendorfs for trypsin digestion according to Shevchenko et al. (142). Briefly, 100 mM ammonium bicarbonate (NH_4HCO_3) and pure acetonitrile (ACN) was added to each gel fraction, with 30-minute between incubations until the gel lose its color. Then, the pieces of gel were reduced with 10 mM dithiothreitol (DTT) at 60ºC and alkylated with 55 mM iodoacetamide (IAA). After incubation with NH_4HCO_3 and ACN, the supernatant was discarded and gel pieces were incubated with trypsin (Pierce, Life Tecnologies, USA) and incubated for 16 hours at 37°C. The peptides were then extracted and lyophilized in a SpeedVac (Thermo Savant).

The tryptic peptides were resuspended in 1% (v/v) formic acid (FA) and analyzed with a QExactive Orbitrap (Thermo Fisher Scientific, Bremen) through the EASY-spray nano ESI source (Thermo Fisher Scientific, Bremen) that was coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA) high-pressure liquid chromatography (HPLC) system. The trap (5 mm × 300 µm internal diameter (ID)) and the EASY-spray analytical (150 mm × 75 µm) columns used were C18 Pepmap100 (Dionex, LC Packings) having a particle size of 3 µm. Peptides were trapped at 30 µL/min in 96 % solvent A (0.1% FA). Elution was achieved with the solvent B (0.1 % FA/80 % acetonitrile v/v) at 300 nL/min. The 92 min gradient used was as follows: 0–3 min, 96 % solvent A; 3–70 min, 4–25 % solvent B; 70–90 min, 25–40 % solvent B; 90–92 min, 90 % solvent B; 90–100 min, 90 % solvent B; 101-120 min, 96 % solvent A. The mass spectrometer was operated at 1.8 kV in the data dependent acquisition mode. A MS^2 method was used with a FT survey scan from 400 to 1600 *m/z* (resolution 70,000; auto gain control (AGC) target 1E6). The 10 most intense peaks were subjected to HCD fragmentation (resolution 17,500; AGC target 5E4, NCE 28 %, max. injection time 100 ms, dynamic exclusion 35 s).

Spectra were processed and analyzed using Proteome Discoverer software version 2.2 (Thermo), with the MS Amanda (version 2.0, University of Applied Sciences Upper Austria, Research Institute of Molecular Pathology) and Sequest HT search engines. Uniprot (TrEMBL and Swiss-Prot) protein sequence database (version of October 2017)

was used for all searches under *Homo sapiens*. Database search parameters were as follows: carbamidomethylation of cysteine, oxidation of methionine, and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 10 ppm and fragment ion mass tolerance was 0.02 Da. To achieve a 1% false discovery rate (FDR), the Percolator (version 2.0, Thermo) node was implemented for a decoy database search strategy and peptides were filtered for high confidence and a minimum length of 6 amino acids, and proteins were filtered for a minimum number of peptide sequences of 1. For each protein, minimal number of identified unique peptides was set to two peptides.

Differences among groups were explored using the free available MetaboAnalyst 4.0 software (143). To determine the biological processes modulated by cachexia, a Cytoscape analysis using the plugins ClueGo+CluePedia (144) was performed. The Funrich (145) and STRING (146) were also used to identify the biological processes more prevalent in the urine of HNC patients, with and without cachexia, and the cellular origin of the proteins identified in the urine.

3.5. Statistical analysis

Values of the serum parameters are presented as mean ± standard deviation for each experimental group. The statistical significance of the differences between groups was determined using the unpaired student t-test. The level of significance was set at 5 % (p-value < 0.05). These statistical analyses were performed with the GraphPad Prism® software for windows (version 7.00).

4. RESULTS

4.1. Characterization of body wasting phenotype of HNC patients

Thirty patients with HNC were included in this study. All patients were male, with an average age of 55 years (range 43 to 77 years). In addition, all patients were smokers or have previously smoked for several years and presented alcoholic habits. Indeed, only one patient never had drinking. Twenty patients reported a body weight loss higher than 5% in 6 months, 93.33 % of patients presented a BMI lower than 25 and from these, 36.67 % lower than 18.5, which is indicative of underweight. To accomplish the goal of the present study, patients were initially grouped according to body weight loss in the last 6 months. The comparative analysis of data retrieved from the analysis of circulating markers of inflammation and catabolism showed no differences among groups (data not shown). Then, HNC patients were grouped according to NRI, which was previously demonstrated to be a good marker to identify subjects at risk of malnutrition (147). Therefore, from the 30 patients enrolled in the present study, 14 were considered cachectic and 16 non-cachectic. No significant age differences were noticed between groups. The anthropometric characteristics and clinical information of patients' groups are presented in Table 3. NRI values are presented only for informative purpose since it was the criterion used for patients' grouping. For the determination of NRI, albumin values and actual and usual weight information were used, so the statistically significant differences observed between groups regarding weight loss and albumin were expected. Regarding BMI, this was lower in patients with cachexia ($p < 0.01$). Patients from HNC group presented an average BMI of 22, which is an indicator of healthy weight (148).

A higher frequency of tongue and pharyngeal tumors (33%) were observed in patients with no cachexia, whereas pharyngeal tumors (50%) were more frequent in cachectic subjects. Concerning the size of the primary tumor (T), a higher percentage (70%) of subjects diagnosed with stage T4 was observed. None of the patients from the HNC+Cachexia group were diagnosed with stage T1. On the other hand, some patients with no signs of cachexia were diagnosed with T1 stage and the percentage of patients with stage T4 was 38%. Regarding regional nodal dissemination (N), patients from both groups presented a higher frequency of stage N2. In the non-cachexia group, most of the subjects were diagnosed with a MUST score of 0. Unlike the observed in this group, in HNC+Cachexia group 25% of patients were identified at high risk of malnutrition once presented a MUST score of 4. To better characterize the association between weight loss

and MUST score, a correlation analysis was performed, and a significant positive association was noticed ($p<0.0001$; Figure 4).

Table *3.* Characterization of HNC patients (grouped according to NRI), relative to age, body weight loss, BMI, location of tumor, tumor staging and MUST score.

		Groups	
		HNC	**HNC+cachexia**
NRI		*104.60 ± 3.12*	*86.60 ± 7.31*****
Age (years)		57.94 ± 7.05	54.86 ± 5.71
Body weight loss (Kg)		4.44 ± 3.87	11.29 ± 6.08**
BMI (Kg/m^2)		21.88 ± 3.53	18.33 ± 2.96**
Location of the tumor, (%)			
Tongue		33.33	25.00
Pharynx		33.33	50.00
Larynx		26.66	0.00
Laryngeal Pharynx		6.67	25.00
Tumor staging, %			
stage T	1	18.75	0.00
	2	0.00	7.69
	3	43.75	23.08
	4	37.50	69.23
stage N	0	31.25	23.00
	1	6.00	0.00
	2	56.25	53.84
	3	6.25	23.07
MUST score, %			
0		57.14	8.33
1		7.14	25.00
2		21.43	33.33
3		14.29	8.33
4		0.00	25.00

*Values are expressed as mean ± standard deviation or percentage. ** p<0.01; **** p<0.0001.*

In addition to the information presented in Table 3, the information about the medication of each patient was made available. Most were analgesics, antihypertensives and some psychotropic drugs appear. Apparently, none of the medication seems to play a significant role in cancer or cachexia. Only the high frequency of analgesics was noticed (data not shown). Although many analgesics cause immunosuppression of innate and

humoral defense systems, which the body relies on for the regulation of cancer development (149), this did not seem a discriminative factor once it appears in both groups.

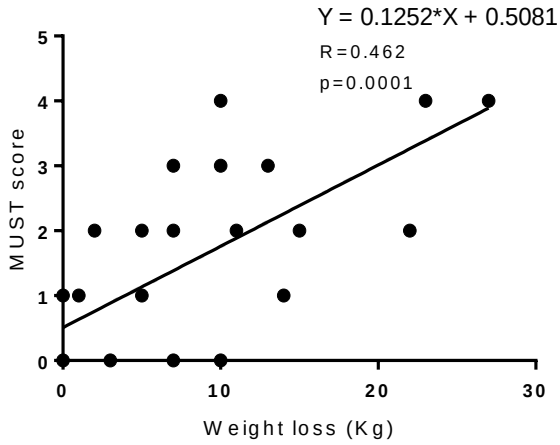

Figure 4. *Correlation between weight loss and MUST score.*

Concerning biochemical parameters assessed in serum samples, albumin levels were significantly distinct between groups ($p < 0.0001$), which was expected since these values were used in NRI calculation and, consequently, in patients' assessment to each group. A significant decrease in cholesterol levels ($p < 0.001$) was observed in patients with cachexia. For the remaining parameters assessed (glucose, urea, and triglycerides), no differences were observed between groups.

Table 4. *Characterization of the serum biochemical profile of patients with HNC grouped according to NRI.*

	Groups	
	HNC	**HNC+cachexia**
Albumin (g/L)	43.25 ± 0.66	34.21 ± 1.49****
Glucose (mmol/L)	5.531 ± 0.203	5.521 ± 0.217
Urea (mmol/L)	4.34 ± 0.394	3.336 ± 0.291
Cholesterol (mmol/L)	4.939 ± 0.248	3.653 ± 0.205***
Triglycerides (mmol /L)	1.334 ± 0.178	1.114 ± 0.092

*Values are expressed as mean ± standard deviation. *** p<0.001; **** p<0.0001.*

4.2. Contribution of inflammatory, catabolic and appetite control processes to HNC-related body wasting

To study the effect of inflammation on the pathogenesis of cachexia in the set of HNC, several parameters were assessed in serum samples collected from patients (Figure 5). The results show that HNC patients with cachexia had significantly higher levels of CRP (Figure 5.A) and of the proinflammatory cytokines IL-6 and TNF-α (Figure 5.B and 5.C, respectively). Despite this pro-inflammatory phenotype, no differences were observed in the content of the metalloproteinases MMP2 and MMP9 (Figure 5.D and 5.E).

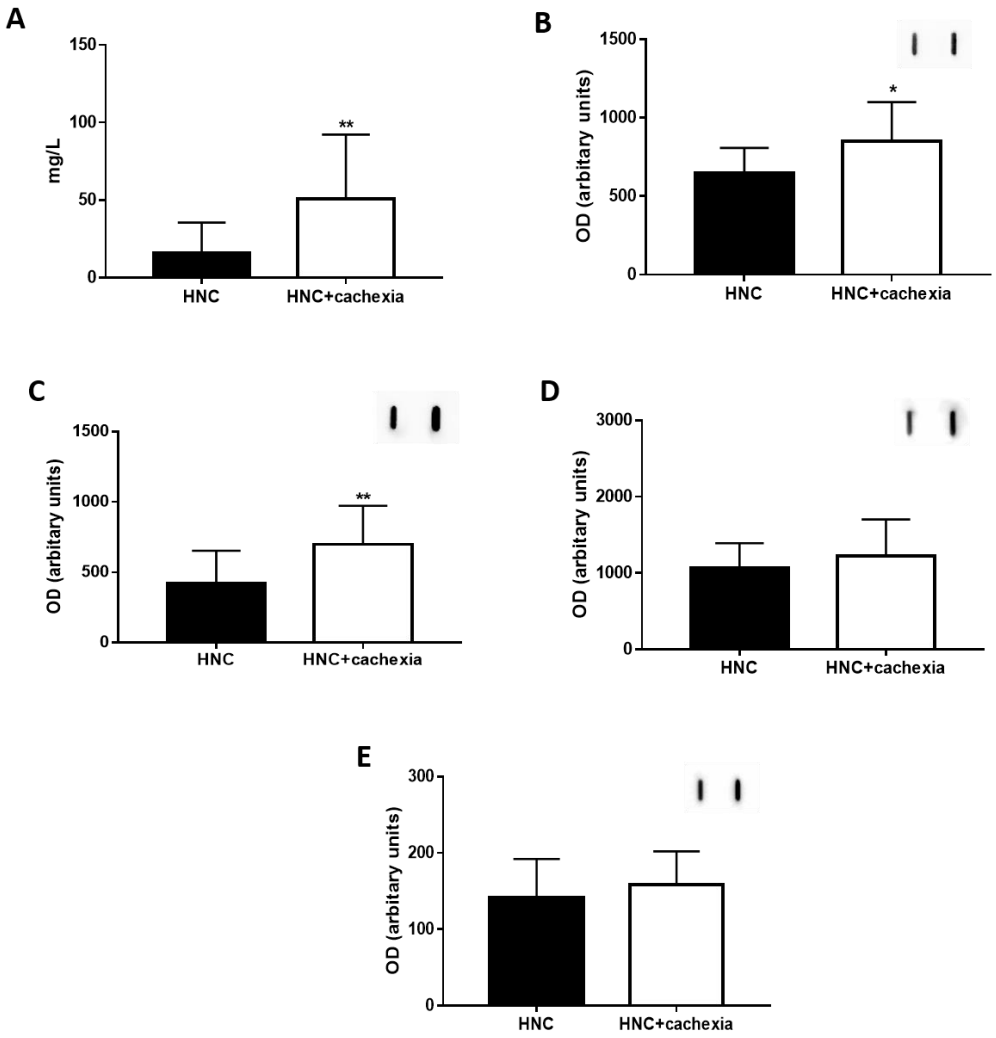

Figure 5. *Evaluation of CRP (A), IL-6 (B), TNF-alpha (C) and metalloproteinases 2 (D) and 9 (E) circulating levels measured by autoanalyzer (in the case of CRP) or immunoblot. A representative image of the obtained blots is shown above the graph. The values (mean ± SD) of the CRP are expressed in mg/L and of the other parameters in arbitrary units of optical density (OD). * p <0.05; ** p <0.01.*

Figure 6 shows the results obtained from the analysis of catabolic and appetite control molecular markers, represented by myostatin (Mstn; Figure 6.A) and ghrelin (Figure 6.B). HNC patients with cachexia presented significantly elevated levels of the myokine Mstn, which may be suggestive of muscle mass loss. Regarding ghrelin, no differences of its circulating levels were observed between groups, suggesting that there were no changes in appetite regulation. However, a negative correlation between ghrelin and CRP was observed (p=0.0056). Data suggest that inflammation is related to decreased appetite (Figure 7).

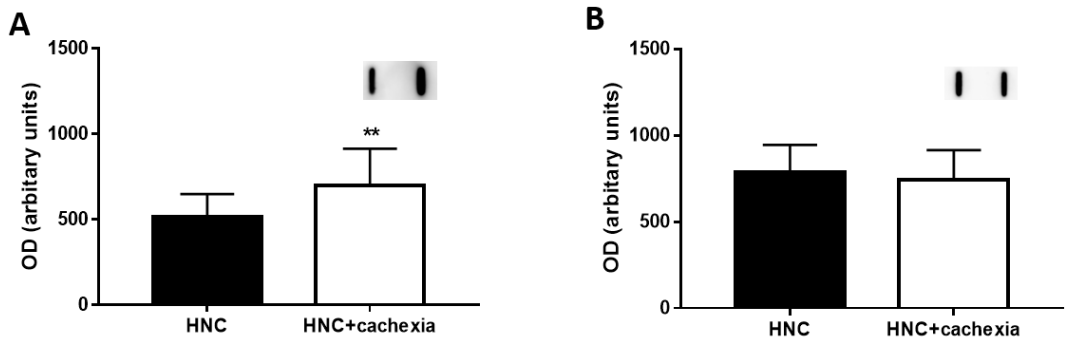

Figure 6. *Levels of the catabolic marker, Mstn (A), and appetite control, ghrelin (B), measured by immunoblot analysis of serum samples. A representative image of the obtained blots is shown above the graph. The values (mean ± SD) are expressed in arbitrary units of optical density (OD). ** p <0.01.*

To better explore this result, patients were tentatively grouped according to body weight loss and CRP values. For patients with body weight loss higher than 5% in less than 6 months and high circulating levels of CRP, significantly lower values of ghrelin were noticed compared to patients with no body weight loss and normal values of CRP (Figure S1).

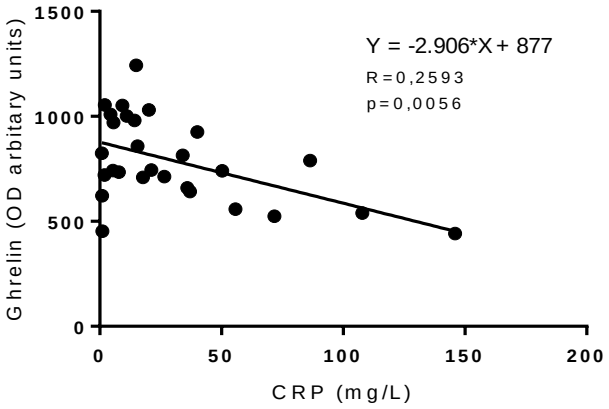

Figure 7. *Correlation between ghrelin and CRP circulating levels.*

4.3. Characterization of urine proteome profile among HNC patients` groups.

The analysis of urine proteome from ten HNC patients, with and without cachexia according to NRI values, allowed the identification of a total of 520 distinct proteins. According to FunRich software (145) most of these proteins belong to “metabolism”, “immune response” and “cell growth and maintenance” biological processes. Regarding the cellular component, most of these proteins are derived from exosomes and of extracellular origin.

The comparative analysis between groups using MetaboAnalyst software (www.metaboanalyst.ca/) highlight a clear urine proteome separation between groups, except for one patient from the HNC+Cachexia group (Figure 8). In fact, a closer analysis of serum markers and clinical data of this patient showed low levels of CRP levels (5.4 mg/L) compared to the other cachectic patients, despite a NRI lower than 97.5, and a normal BMI value.

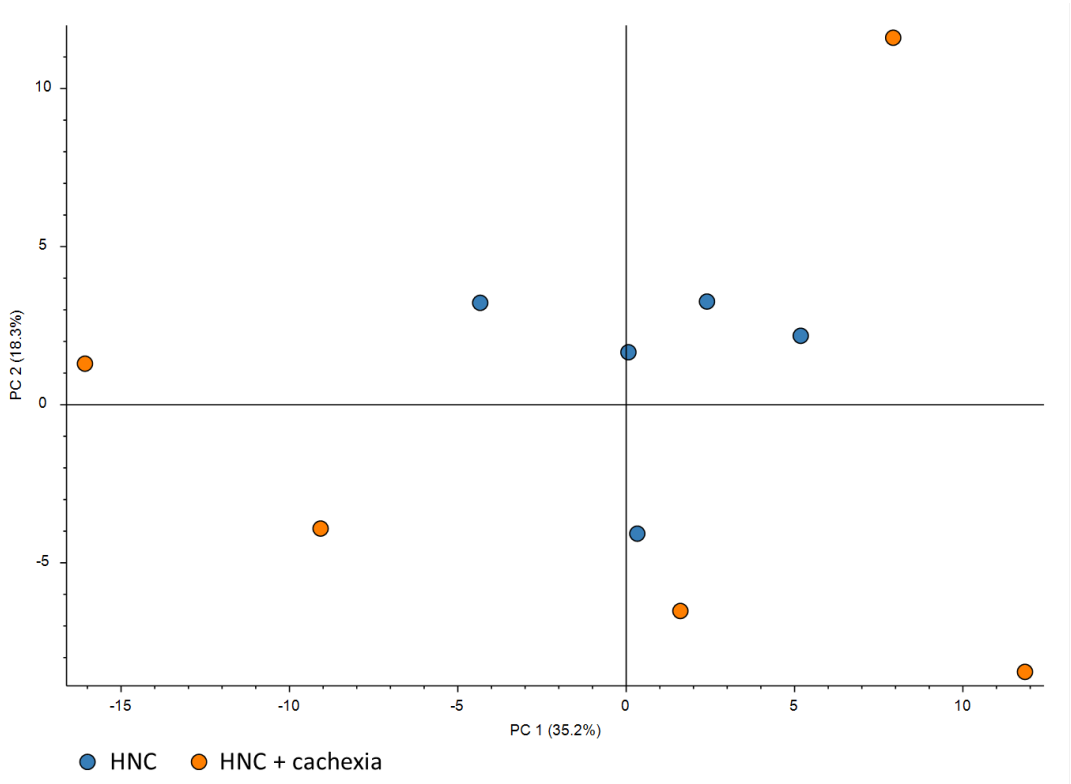

Figure 8. *Principal Component Analysis plot of the proteins present in the urine from the two groups of patients.*

Looking to the proteins present in significant distinct amounts between groups, 18 proteins were identified (Table 5). Eleven proteins were present in significant higher (pink table lines) levels in the urine of cachectic patients whereas seven were in significant lower (blue table lines) amounts.

The analysis of the biological processes with the ClueGo+CluePedia plugins from Cytoscape software considering the proteins present in significant higher levels in HNC+Cachexia group highlighted the contribution of "ROS metabolism" and the "regulation of leukocytes metabolism" whereas the lower abundant proteins belong to "hydrogen peroxide catabolism process". Among the proteins found in higher amounts in the urine of cachectic patients are CRP, which highlight the contribution of inflammation to cachexia pathogenesis, further supporting data from serum analysis. The enzyme argininosuccinate synthase was previously associated to HNC (150). Also, cornulin, small proline-rich protein 3 and cystatin-B have been previously associated with HNC (151,152). Regarding cornulin, loss of its expression seems to be associated with the development of local relapse (151). Small proline-rich protein 3 was associated with

increased sensitivity of cancer cells in response to DNA damage-induced apoptosis (153). However, no prior association of these proteins to cachexia pathogenesis was found. The C4b-binding protein alpha chain was previously associated to the positive regulation of the catabolic process (154), supporting its putative involvement in cachexia. Carboxymethylenebutenolidase homolog was involved to in the differentiation of myoblasts, being implicated in the differentiation and responsiveness of myoblasts to TNF-α (155).

Table 5. *Comparative quantitative analysis of urine proteome between groups. The proteins at the bottom of the table (pink) are the ones present in higher amounts in the urine of HNC patients with cachexia. The proteins at the top of the table (blue) are proteins present in higher amounts in the urine of non-cachectic HNC patients.*

Accession number (Uniprot)	Name	Coverage [%]	Peptides	MW [kDa]	pI	Abundance Ratio: (HNC+Cach) / (HNC)	P-Value: (HNC+Cach) / (HNC)
P14780	Matrix metalloproteinase-9	4	2	78.4	6.06	0.01	3.02E-16
P02042	Hemoglobin subunit delta	45	7	16	8.05	0.01	3.02E-16
P55291	Cadherin-15	16	8	88.9	4.98	0.179	0.001762
Q8N307	Mucin-20	26	2	71.9	5.07	0.198	0.004114
Q66K79	Isoform 2 of Carboxypeptidase Z	3	2	72.5	8.09	0.243	0.020615
Q6UX06	Olfactomedin-4	40	16	57.2	5.69	0.25	0.02573
P05164	Isoform H7 of Myeloperoxidase	6	4	87.2	9.07	0.267	0.040073
Q9UBG3	Cornulin	20	4	53.5	6.1	4.299	0.040069
Q96DG6	Carboxymethylenebutenolidase homolog	13	2	28	7.18	4.604	0.02434
P16083	Ribosyldihydronicotinamide dehydrogenase [quinone]	13	2	25.9	6.29	4.758	0.019244
P00966	Argininosuccinate synthase	5	2	46.5	8.02	4.799	0.018177
P04003	C4b-binding protein alpha chain	6	3	67	7.3	5.087	0.011475
P02741	C-reactive protein	9	2	25	5.63	5.153	0.010451
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	10	6	106.4	6.86	6.03	0.002888
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	3	3	99.8	5.74	8.422	0.000132
Q6UWR7	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6 [OS=Homo sapiens]	8	3	50.2	7.99	100	3.02E-16
P04080	Cystatin-B [OS=Homo sapiens]	37	2	11.1	7.56	100	3.02E-16
Q9UBC9	Small proline-rich protein 3 [OS=Homo sapiens]	18	2	18.1	8.57	100	3.02E-16

5. DISCUSSION

The high rates of morbidity and mortality in patients with HNC are closely associated with critical weight loss, a common feature in these patients (156). Weight loss is greater and has a higher incidence in HNC patients compared to other cancer patients because, at least in part, to the difficulty in swallowing presented by these patients (157). Smoking and drinking habits, the most common etiological factors associated to the development of HNC, has also been associated to malnutrition (157). Indeed, Martin Villares et al. (158) showed that the risk of malnutrition increased with the amount of alcohol consumed. One of the hypotheses raised to explain this association is the thiamine deficiency evidenced by patients with alcohol dependence, which seems to result in poor oral absorption (159). However, several studies associate HNC with cachexia (42,160), a syndrome characterized by muscle loss not reverted by parental nutrition (41). The clinical management of cachexia is complex and rarely recognized or assessed (161). Indeed, more than 80 clinical trials have been conducted to assess the putative anti-cancer cachexia effect of proposed therapeutic strategies (**www.clinicaltrials.go)** but no successful results were reported. This may be justified, at least in part, by the misdiagnosis of cachexia in the set of HNC. To overcome this gap, we applied a proteomic strategy to characterize body fluids collected from HNC patients with and without a wasting phenotype. The biggest challenge that we faced was how to group patients with (HNC+Cachexia group) and without cachexia (HNC group). The Nutritional Risk Index (NRI) was the index chosen because it is highly sensitive when compared to other nutritional assessments. In the investigation of the ability to detect conditions such as sarcopenia, myosteatosis, pre-cachexia, and cancer cachexia, NRI presented the better accuracy (about 74.7%) in identifying patients with at least one of these conditions, when compared to other screening tools (e.g. MST, Malnutrition Screening Tool; MUST, Malnutrition Universal Screening Tool) (162).

Once established the experimental groups, we performed a targeted proteomic analysis of serum samples. Several protein targets were selected, specifically inflammatory, catabolic and appetite control markers. One of the main features of cachexia is inflammation (43). The presence of an inflammatory state is one of the points that distinguishes cachexia from other syndromes such as sarcopenia (52,163); however, inflammation is always present in HNC, even if not at very high levels (164). So, one of the focus of this study was the link between inflammation and body weight loss. Although

non-specific, CRP is a sensitive marker of inflammation. Its synthesis by hepatocytes is induced by proinflammatory cytokines, mainly IL-6. Our results show a significant increase of serum CRP and IL-6 in patients with cachexia (Figure 5). The persistence of an inflammatory state results in the continuous production of this acute phase protein, promoting the reprioritization of amino acid metabolism in peripheral tissues, especially muscles, towards the liver. Therefore, muscle catabolism is expected to occur (165). The increase of circulating CRP levels in cachectic patients was paralleled by the increase of IL-6 (Figure 5), which is known to promote the liver synthesis of CRP (165).

Besides IL-6, circulating TNF-α levels were previously associated with HNC (59,84). Our results evidenced increased levels of these cytokines in the group of patients with cachexia (Figure 5), reinforcing the contribution of inflammation to the wasting phenotype. Moreover, higher levels of inflammatory markers have been associated with lower strength and muscle mass (166). This appears to be explained by the fact that IL-6 activates the JAK/STAT3 signaling pathway in skeletal muscle, leading to the UPP activation and inhibition of the PI3K/Akt/mTOR pathway (64). On the other hand, TNF-α activates the NF-κB and p38 MAPK pathways, both of which induce the upregulation of the major E3 ligases (MuRF1 and atrogin) that mediate muscle mass loss and protein synthesis inhibition (113).

The contribution of muscle catabolism to the HNC-related wasting phenotype was evaluated through the analysis of circulating myostatin. Since myostatin is secreted and expressed mainly by the skeletal muscle, its main function is the negative regulation of muscle mass (blocking myoblast proliferation and suppression of satellite cell activation) (167). In fact, studies demonstrate that myostatin is implicated in various diseases involving muscle mass loss (168,169). Data obtained highlight a significant increase of this protein in the serum from patients with cachexia (Figure 6). A negative correlation has already been made between elevated myostatin serum levels and skeletal muscle mass (170). Therefore, this result suggests the involvement of muscle mass loss in the pathogenesis of cachexia. The role of myostatin in the regulation of muscle mass involves the activation of activin type I and II receptors (125). Thus, these receptors may be seen as putative therapeutic targets. In fact, BY338 (131) and REGN10 (132), two monoclonal antibodies, were shown to inhibit the activin type II receptor with the consequent preservation of muscle mass; however, studies were only done on pre-clinical models.

Appetite dysregulation has been suggested to contribute to body weight loss in cancer (171). Ghrelin, mainly produced by the entero-endocrine cells of the stomach (125), was the marker selected in our study for the analysis of the contribution of appetite regulation to cachexia pathogenesis. This hormone binds to the GH receptor and stimulates appetite via neuropeptide Y activation in the hypothalamus (125). No significant variations of ghrelin levels were observed among the studied groups (Figure 6), which suggests that appetite control is not a good therapeutic target for the management of HNC cachexia. However, a significant negative correlation was observed between ghrelin and CRP (Figure 7). Thus, patients with high levels of CRP (above 30 mg/L) may benefit from the therapeutic use of ghrelin analogs. Indeed, there are clinical trials reporting positive results for the use of ghrelin analogs in the regulation of body composition and weight gain. Ghrelin analogs have been preferred to ghrelin because the use of the hormone was associated to many severe adverse events, such as acute myocardial infarction (172). Anamorelin is an analog of ghrelin used in several studies and despite the good results in terms of nutritional status and increase in lean mass, the results related to muscle function are contradictory (173,174).

CRP and ghrelin results reinforce the importance of the criteria used to group patients on data variation of selected parameters. Indeed, despite being considered cachectic according to the NRI, some of the patients presented low levels of inflammation, which is not in agreement with the previously supposed about the contribution of inflammation to cachexia. These results raised some curiosity and interest in the study of a possible third group of patients (Figure S1) with characteristics like weight loss, but without inflammation. Using this criterion, it is clear the association between inflammation and appetite dysregulation. Thus, efforts should continue to be made to promote a better diagnosis of cachexia and, consequently, improve the selection of therapeutic approaches.

In order to explore the diagnosis value of urine in cancer cachexia and to identify novel markers of this syndrome, we applied a high throughput MS-based approach to characterize the urine proteome from HNC patients with and without cachexia (according to the NRI score). Compared to blood-derived fluids, urine has several advantages such as its non-invasively collection in unrestricted amounts. In addition, urine proteome is not dominated by highly abundant proteins. Indeed, serum proteome is dominated by 22

(highly abundant) proteins that make up 99% of the total protein mass, with half of this mass represented by albumin (175). Urine proteome is less complex, containing approximately 2000 proteins against more than 10000 of the plasma (176).

Using a GeLC-MS/MS approach we identified 520 distinct proteins (each with 2 peptides, FDR lower than 1%). Within the set of the identified proteins are commonly found urinary proteins such as uromodulin (177), immunoglobulins (178) and osteopontin (179). In overall, most of the identified proteins belong to the biological processes "metabolism", "immune response" and "cell growth and maintenance". The “immune response” and “metabolism” draw attention within the scope of this work since the progression of cancer and cachexia depends on the immune response. The metabolism of neoplastic cells may have contributed to the urine proteome of HNC patients. Altered metabolic activity related to lipolysis, the TCA cycle, and amino acid catabolism was previously reported in the set of HNC (180). HNC tumors affect the metabolism of their hosts in such a way that it can most often result in cachexia (181). At the same time, the cachexia itself affects much of the metabolism of the human body (113).

The comparative analysis of urine proteome between patients with and without cachexia showed 18 proteins in significant different amounts. The analysis of the biological processes involving these proteins highlighted "ROS metabolism" and "regulation of leukocyte metabolism". CRP was present in higher amounts in the urine of cachectic patients confirming the results obtained in the serum analysis. C4b binding protein alpha chain, another protein found in greater amounts in the urine of cachectic patients, is also an acute phase protein (182) that belongs to the complement system (183). These results also reinforce the contribution of inflammation to the development of cachexia. Other proteins identified in higher amounts in the urine of cachectic patients were previously associated with HNC, including the enzyme argininosuccinate synthase (150), cornulin, small proline-rich protein 3 and cystatin B (151,152). Increased urinary and serum levels of argininosuccinate synthase were reported to be associated with inflammation (184). Cornulin and the small proline-rich protein 3 make part of the protective barrier of the mucosa and the skin (151). Previous studies showed a low expression of these proteins in the tumor tissue from HNC patients (151,152), possibly justifying the increased content observed in the urine of patients at advanced stage of the disease. However, the negative regulation of cornulin has been reported in several types of cancer and associated with advanced clinical stages, making difficult to comprehend

its increase in the urine of HNC patients with cachexia. Cystatin-B, a cathepsin-B inhibitor, was also found to be diminished in HNSCC (tissue samples) (152), unlike the reported in the set of bladder cancer (185). To the best of our knowledge, ribosyldihydronicotinamide dehydrogenase, a protein that confers protection against oxidative stress and carcinogenesis (186), was not previously associated to HNC neither to cachexia. Future studies will be important to increase the number of urine samples analyzed and to validate these putative HNC cachexia biomarkers such as CRP, C4-binding protein alpha chain and argininosuccinate synthase.

2. CONCLUSION AND FUTURE PERSPECTIVES

Cachexia is a common problem among patients with HNC; however, this syndrome is often misdiagnosed despite its negative impact on disease outcomes. To add new insights on this issue, targeted and non-targeted proteomics analysis of body fluids from 30 HNC patients was performed and the results obtained were correlated with clinical information. Data integration allowed us to take the following conclusions:

i) The diagnosis of cachexia based on body weight loss higher than 5% in less than 6 months does not allow the discrimination of HNC patients with laboratory biochemical parameters. NRI seems to be a better assessment tool of cachexia in the set of HNC;

ii) Cachexia (based on an NRI lower than 97.5) is associated to advanced stages of disease, high degree of inflammation, given by increased levels of CRP, TNF-α, and IL-6, and muscle catabolism, which is related to high levels of circulating myostatin;

iii) The negative correlation observed between circulating levels of ghrelin and CRP suggests that patients with CRP levels higher than 30 mg/dL may benefit from therapy with ghrelin agonists;

iv) Urine proteomics confirmed the diagnosis value of this body fluid since a clear separation of proteome profile was observed among HNC patients with and without cachexia;

v) Eleven proteins were found in higher levels in the urine of cachectic patients, being of notice C4 binding protein alpha chain, CRP and argininosuccinate synthase, which emphasizes the contribution of inflammation to HNC cachexia pathogenesis.

Future studies will be important to increase the number of samples analyzed and to validate the putative biomarkers identified, envisioning the improvement of cachexia diagnosis in the set of HNC and, eventually, in other cancer types. We believe that more than one biomarker, a multimarker panel combining several protein targets will better approach the cachexia diagnosis, improving the management of this syndrome and, consequently, disease prognosis.

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8. SUPPLEMENTARY DATA

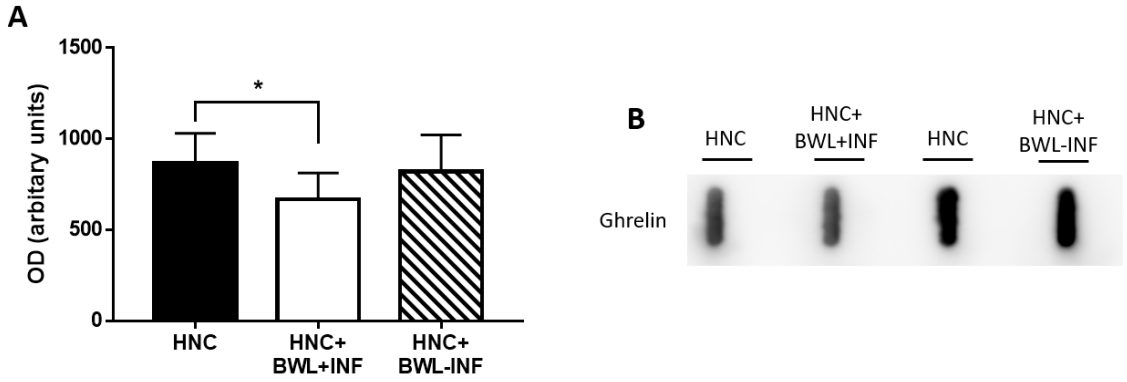

Figure S1. *Levels of expression of the appetite control parameter, ghrelin (A), measured by immunoblot, of patients with HNC, without weight loss and without inflammation (HNC), with weight loss and inflammation (HNC+BWL+INF) and with weight loss and without inflammation (HNC+BWL-INF). A representative immunoblot is presented in (B). The values (mean ± SD) are expressed in arbitrary units of optical density (OD). * p <0.05.*

Table S1. *Reference values of the serum parameters provided by the IPO.*

Laboratory parameter	Reference values
Albumin	38-53 (g/L)
Glucose	4.2-6.4 (mmol/L)
Urea	1.6-8.3 (mmol/L)
Cholesterol	<5.18 (mmol/L)
Triglycerides	0.5-1.71 (mmol/L)

***Table S2**. List of proteins identified in the GeLC-MS/MS analysis of urine from 10 HNC patients (520 different proteins).*

Accession	Description	Coverage [%]	Peptides	MW [kDa]	calc. pI	Abundance Ratio: (Sample) / (Control)	P-Value: (Sample) / (Control)
Q6UWR7	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6 [OS=Homo sapiens]	8	3	50.2	7.99	100	3.02E-16
P04080	Cystatin-B [OS=Homo sapiens]	37	2	11.1	7.56	100	3.02E-16
P14780	Matrix metalloproteinase-9 [OS=Homo sapiens]	4	2	78.4	6.06	0,01	3.02E-16
Q9UBC9	Small proline-rich protein 3 [OS=Homo sapiens]	18	2	18.1	8,57	100	3.02E-16
P02042	Hemoglobin subunit delta [OS=Homo sapiens]	45	7	16	8.05	0.01	3.02E-16
Q06033-1	Inter-alpha-trypsin inhibitor heavy chain H3 [OS=Homo sapiens]	3	3	99.8	5.74	8.422	0.000132
P55291	Cadherin-15 [OS=Homo sapiens]	16	8	88.9	4.98	0.179	0.001762
P19823	Inter-alpha-trypsin inhibitor heavy chain H2 [OS=Homo sapiens]	10	6	106.4	6.86	6.03	0.002888
Q8N307	Mucin-20 [OS=Homo sapiens]	26	2	71.9	5.07	0.198	0.004114
P02741-1	C-reactive protein [OS=Homo sapiens]	9	2	25	5.63	5.153	0.010451
P04003	C4b-binding protein alpha chain [OS=Homo sapiens]	6	3	67	7.3	5.087	0.011475
P00966	Argininosuccinate synthase [OS=Homo sapiens]	5	2	46.5	8.02	4.799	0.018177
P16083	Ribosyldihydronicotinamide dehydrogenase [quinone] [OS=Homo sapiens]	13	2	25.9	6.29	4.758	0.019244
Q66K79-2	Isoform 2 of Carboxypeptidase Z [OS=Homo sapiens]	3	2	72.5	8.09	0.243	0.020615
Q96DG6	Carboxymethylenebutenolidase homolog [OS=Homo sapiens]	13	2	28	7.18	4.604	0.02434
Q6UX06	Olfactomedin-4 [OS=Homo sapiens]	40	16	57.2	5.69	0.25	0.02573
Q9UBG3	Cornulin [OS=Homo sapiens]	20	4	53.5	6.1	4.299	0.040069
P05164-3	Isoform H7 of Myeloperoxidase [OS=Homo sapiens]	6	4	87.2	9.07	0.267	0.040073
P48735	Isocitrate dehydrogenase [NADP], mitochondrial [OS=Homo sapiens]	8	3	50.9	8.69	3.643	0.085729
Q95604	HLA class I histocompatibility antigen, Cw-17 alpha chain [OS=Homo sapiens]	10	2	41.2	6.8	0.314	0.85729
P05546	Heparin cofactor 2 [OS=Homo sapiens]	5	3	57	6.9	3.587	0.090394
Q9HBJ8	Collectrin [OS=Homo sapiens]	13	2	25.2	5.63	3.574	0.091971

P0DOX3	immunoglobulin delta heavy chain [OS=Homo sapiens]	6	2	56.2	8.02	3.535	0.098044
P0C0L4-1	Complement C4-A [OS=Homo sapiens]	32	39	192.7	7.08	0.35	0.148373
P07900-2	Isoform 2 of Heat shock protein HSP 90-alpha [OS=Homo sapiens]	7	5	98.1	5.16	3.27	0.148373
P52823	Stanniocalcin-1 [OS=Homo sapiens]	13	2	27.6	7.99	3.222	0.159845
P02792	Ferritin light chain [OS=Homo sapiens]	18	3	20	5.78	0.356	0.161447
Q9BTY2	Plasma alpha-L-fucosidase [OS=Homo sapiens]	6	2	54	6.25	3.144	0.18097
P08571	Monocyte differentiation antigen CD14 [OS=Homo sapiens]	43	13	40.1	6.23	0.368	0.191799
Q6UXB8-1	Peptidase inhibitor 16 [OS=Homo sapiens]	22	7	49.4	5.39	0.37	0.194171
P07686	Beta-hexosaminidase subunit beta [OS=Homo sapiens]	22	10	63.1	6.76	2.927	0.250525
P13671	Complement component c6 [OS=Homo sapiens]	4	2	104.7	6.76	2.887	0.264851
P25311	Zinc-alpha-2-glycoprotein [OS=Homo sapiens]	58	22	34.2	6.05	0.403	0.289474
P05062	fructose-bisphosphate aldolase B [OS=Homo sapiens]	39	10	39.4	7.87	2.835	0.289474
P09668	Pro-cathepsin H [OS=Homo sapiens]	12	4	37.4	8.07	2.833	0.289474
P30039	Phenazine biosynthesis-like domain-containing protein [OS=Homo sapiens]	10	2	31.8	6.52	2.828	0.289474
P04196	Histidine-rich glycoprotein [OS=Homo sapiens]	11	4	59.5	7.5	2.821	0.291738
P12273	Prolactin-inducible protein [OS=Homo sapiens]	33	5	16.6	8.05	0.408	0.302855
P80188	Neutrophil gelatinase-associated lipocalin [OS=Homo sapiens]	46	7	22.6	8.91	0.413	0.315967
P12882	Myosin-1 [OS=Homo sapiens]	5	6	223	5.74	0.416	0.315967
P02760	Protein AMBP [OS=Homo sapiens]	52	19	39	6.25	0.423	0.345973
Q9Y653	Adhesion G-protein coupled receptor G1 [OS=Homo sapiens]	3	2	77.7	8.48	0.429	0.357041
P20711	aromatic-L-amino-acid decarboxylase [OS=Homo sapiens]	5	2	53.9	7.2	2.648	0.360998
Q04756	Hepatocyte growtH factor activator [OS=Homo sapiens]	3	2	70.6	7.24	0.437	0.385481
P02765	Alpha-2-HS-glycoprotein [OS=Homo sapiens]	40	10	39.3	5.72	0.442	0.399838
Q96PD5-2	Isoform 2 of N-acetylmuramoyl-L-alanine amidase [OS=Homo sapiens]	23	10	68	7.68	0.447	0.419337
P04792	Heat shock protein beta-1 [OS=Homo sapiens]	21	3	22.8	6.4	2.542	0.426764
P19961	Alpha-amylase 2B [OS=Homo sapiens]	50	17	57.7	7.09	0.453	0.434403

P05543	thyroxine-binding globulin [OS=Homo sapiens]	34	11	46.3	6.3	0.463	0.475057
P05787-2	Isoform 2 of Keratin, type II cytoskeletal 8 [OS=Homo sapiens]	7	3	56.6	5.43	2.462	0.479089
P02743	Serum amyloid P-component [OS=Homo sapiens]	26	6	25.4	6.54	0.471	0.510173
P34896-1	Serine hydroxymethyltransferase, cytosolic [OS=Homo sapiens]	9	3	53	7.71	2.421	0.510173
P51884	Lumican [OS=Homo sapiens]	14	3	38.4	6.61	0.472	0.510173
Q9Y2S2-1	Lambda-crystallin homolog [OS=Homo sapiens]	11	3	35.4	6.18	2.395	0.532624
P04075	fructose-bisphosphate aldolase A [OS=Homo sapiens]	13	3	39.4	8.09	2.356	0.544506
Q9H0W9	Ester hydrolase C11orf54 [OS=Homo sapiens]	13	3	35.1	6.7	2.361	0.544506
O00533-2	Isoform 2 of Neural cell adhesion molecule L1-like protein [OS=Homo sapiens]	2	2	136.6	5.8	2.356	0.544506
O94919	endonuclease domain-containing 1 protein [OS=Homo sapiens]	34	11	55	5.71	0.488	0.557938
P02788	Lactotransferrin [OS=Homo sapiens]	56	28	78.1	8.12	0.489	0.55876
Q9Y6R7	IgGFc-binding protein [OS=Homo sapiens]	6	12	571.6	5.34	0.494	0.57726
P0DJD8	Pepsin A-3 [OS=Homo sapiens]	9	4	42	4.41	0.495	0.578242
P52565	rho GDP-dissociation inhibitor 1 [OS=Homo sapiens]	16	2	23.2	5.11	2.305	0.578242
P50995	annexin A11 [OS=Homo sapiens]	12	5	54.4	7.65	0.497	0.579995
P09467	Fructose-1,6-bisphosphatase 1 [OS=Homo sapiens]	33	7	36.8	6.99	2.275	0.607632
P06702	Protein S100-A9 [OS=Homo sapiens]	25	2	13.2	6.13	0.509	0.640344
P05451	Lithostathine-1-alpha [OS=Homo sapiens]	48	7	18.7	5.94	0.512	0.643973
P68032	Actin, alpha cardiac muscle 1 [OS=Homo sapiens]	35	9	42	5.39	2.236	0.643973
P00749	Urokinase-type plasminogen activator [OS=Homo sapiens]	42	13	48.5	8.41	0.516	0.647982
Q9BRK3-2	Isoform 2 of Matrix remodeling-associated protein 8 [OS=Homo sapiens]	23	9	49.6	6.92	0.522	0.657939
P69905	Hemoglobin subunit alpha [OS=Homo sapiens]	58	6	15.2	8.68	0.519	0.657939
Q14767	Latent-transforming growth factor beta-binding protein 2 [OS=Homo sapiens]	2	3	194.9	5.19	0.522	0.657939
Q9BUT1-1	3-hydroxybutyrate dehydrogenase type 2 [OS=Homo sapiens]	10	2	26.7	7.65	2.195	0.657939
P39059	Collagen alpha-1(XV) chain [OS=Homo sapiens]	6	6	141.6	5	0.527	0.673338
Q8WVN6	Secreted and transmembrane protein 1 [OS=Homo sapiens]	22	4	27	7.43	0.527	0.673338

P08603-1	complement factor H [OS=Homo sapiens]	6	6	139	6.61	2.15	0.6826
Q15113	Procollagen C-endopeptidase enhancer 1 [OS=Homo sapiens]	13	4	47.9	7.43	0.534	0.694995
P07339	Cathepsin D [OS=Homo sapiens]	48	15	44.5	6.54	2.12	0.715767
P26038	Moesin [OS=Homo sapiens]	15	8	67.8	6.4	2.093	0.746021
P01024	Complement C3 [OS=Homo sapiens]	40	51	187	6.4	2.092	0.746284
Q15274	nicotinate-nucleotide pyrophosphorylase [carboxylating] [OS=Homo sapiens]	9	2	30.8	6.21	2.087	0.749057
Q9UKU9	angiopoietin-related protein 2 [OS=Homo sapiens]	19	8	57.1	7.53	0.55	0.753713
P55287-1	Cadherin-11 [OS=Homo sapiens]	10	5	87.9	4.91	0.551	0.753713
P60709	Actin, cytoplasmic 1 [OS=Homo sapiens]	42	11	41.7	5.48	2.064	0.765022
P06396	Gelsolin [OS=Homo sapiens]	28	11	85.6	6.28	0.559	0.776505
Q93088	Betaine--homocysteine S-methyltransferase 1 [OS=Homo sapiens]	40	10	45	7.03	2.041	0.776505
P08185	corticosteroid-binding globulin [OS=Homo sapiens]	26	8	45.1	6.04	0.56	0.776505
P04180	phosphatidylcholine-sterol acyltransferase [OS=Homo sapiens]	13	5	49.5	6.11	0.558	0.776505
P02675	Fibrinogen beta chain [OS=Homo sapiens]	45	16	55.9	8.27	2.029	0.788034
O75882-1	Attractin [OS=Homo sapiens]	13	13	158.4	7.31	0.567	0.7929
P10643	Complement component C7 [OS=Homo sapiens]	12	7	93.5	6.48	2.002	0.80048
P62258-1	14-3-3 protein epsilon [OS=Homo sapiens]	17	4	29.2	4.74	1.994	0.811171
Q13510-2	Isoform 2 of Acid ceramidase [OS=Homo sapiens]	36	12	46.5	7.85	1,972	0.82223
Q96KP4	cytosolic non-specific dipeptidase [OS=Homo sapiens]	35	11	52.8	5.97	1.932	0.82223
O00468-3	Isoform 3 of Agrin [OS=Homo sapiens]	6	7	215.2	6.51	0.585	0.82223
P24855	Deoxyribonuclease-1 [OS=Homo sapiens]	43	6	31.4	4.91	0,596	0.82223
Q12794-1	Hyaluronidase-1 [OS=Homo sapiens]	18	5	48.3	6.77	0.592	0.82223
P08519	apolipoprotein(a) [OS=Homo sapiens]	19	4	501	5.88	0.588	0.82223
P29508	Serpin B3 [OS=Homo sapiens]	14	4	44.5	6.81	0.582	0.82223
P0DMV8	heat shock 70 kDa protein 1A [OS=Homo sapiens]	9	4	70	5.66	1.973	0.82223
P59665	Neutrophil defensin 1 [OS=Homo sapiens]	19	2	10.2	6.99	0.578	0.82223
Q99969	Retinoic acid receptor responder protein 2 [OS=Homo sapiens]	14	2	18.6	9.09	0.581	0.82223

O14745	Na(+)/H(+) exchange regulatory cofactor NHE-RF1 [OS=Homo sapiens]	8	2	38.8	5.77	1.939	0.82223
P58499-2	Isoform A of Protein FAM3B [OS=Homo sapiens]	9	2	30.4	9	0.592	0.82223
P01009-1	alpha-1-antitrypsin [OS=Homo sapiens]	56	24	46.7	5.59	1.898	0.837154
P04746	Pancreatic alpha-amylase [OS=Homo sapiens]	59	19	57.7	7.05	0.602	0.837154
P23470	Receptor-type tyrosine-protein phosphatase gamma [OS=Homo sapiens]	3	4	161.9	642	0.601	0.837154
P04216	Thy-1 membrane glycoprotein [OS=Homo sapiens]	24	3	17.9	8.73	0.602	0.837154
P06732	Creatine kinase M-type [OS=Homo sapiens]	18	5	43.1	7.25	0.608	0.848378
Q9HCU0	endosialin [OS=Homo sapiens]	15	7	80.8	5.35	0.609	0.852323
P51688	N-sulphoglucosamine sulphohydrolase [OS=Homo sapiens]	28	10	56.7	6.95	1.865	0.869906
P01602	Immunoglobulin kappa variable 1-5 [OS=Homo sapiens]	29	2	12.8	8.28	0.615	0.875985
P04083	annexin A1 [OS=Homo sapiens]	36	9	38.7	7.02	1.853	0.881084
Q9BYF1	angiotensin-converting enzyme 2 [OS=Homo sapiens]	3	2	92.4	5.54	1.847	0.881084
P26842	CD27 antigen [OS=Homo sapiens]	16	2	29.1	7.64	1.853	0.881084
P68363	Tubulin alpha-1B chain [OS=Homo sapiens]	20	6	50.1	5.06	1.826	0.88414
P01861	Immunoglobulin heavy constant gamma 4 [OS=Homo sapiens]	49	10	35.9	736	1.836	0.88414
Q9H6X2	Anthrax toxin receptor 1 [OS=Homo sapiens]	8	4	62.7	7.61	0.626	0.88414
Q9HCN6-3	Isoform 3 of Platelet glycoprotein VI [OS=Homo sapiens]	6	3	67.4	879	0.624	0.88414
O43653	prostate stem cell antigen [OS=Homo sapiens]	23	3	12.9	5.29	0.624	0.88414
P27105	erythrocyte band 7 integral membrane protein [OS=Homo sapiens]	11	2	31.7	7.88	1.822	0.88912
P14550	alcohol dehydrogenase [NADP(+)] [OS=Homo sapiens]	16	3	36.6	6.79	1.811	0.898654
P12277	Creatine kinase B-type [OS=Homo sapiens]	10	3	42.6	5.59	1.809	0.900497
P80748	Immunoglobulin lambda variable 3-21 [OS=Homo sapiens]	39	2	12.4	5.29	1.804	0.908235
P07288-1	Prostate-specific antigen [OS=Homo sapiens]	73	10	28.7	7.68	0.642	0.912559
O00187-1	Mannan-binding lectin serine protease 2 [OS=Homo sapiens]	13	10	75.7	5.63	0.637	0.912559
P08582-1	Melanotransferrin [OS=Homo sapiens]	17	9	80.2	5.94	0.644	0.912559
Q9UHL4	Dipeptidyl peptidase 2 [OS=Homo sapiens]	17	7	54.3	6.32	1.77	0.912559

P05452	Tetranectin [OS=Homo sapiens]	35	6	22.5	5.67	0.635	0.912559
P13473-3	Isoform LAMP-2C of Lysosome-associated membrane glycoprotein 2 [OS=Homo sapiens]	7	3	45.1	5.91	1.778	0.912559
P98095-2	Isoform 2 of Fibulin-2 [OS=Homo sapiens]	3	3	131.8	4.86	0.638	0.912559
Q15485-1	Ficolin-2 [OS=Homo sapiens]	15	3	34	6.77	0.645	0.912559
P00338-3	Isoform 3 of L-lactate dehydrogenase A chain [OS=Homo sapiens]	9	3	39.8	8.43	1.772	0.912559
P52758	2-iminobutanoate/2-iminopropanoate deaminase [OS=Homo sapiens]	34	3	14.5	8.68	1.765	0.919652
P15121	aldose reductase [OS=Homo sapiens]	8	2	35.8	6.98	0.651	0.931572
P41222	Prostaglandin-H2 D-isomerase [OS=Homo sapiens]	35	4	21	7.8	0.651	0.931674
P07858	Cathepsin B [OS=Homo sapiens]	25	6	37.8	6.3	1.752	0.931795
Q16769	glutaminyl-peptide cyclotransferase [OS=Homo sapiens]	43	9	40.9	6.61	0.661	0.931917
P02766	Transthyretin [OS=Homo sapiens]	65	7	15.9	5.76	0.661	0.931917
Q02818	Nucleobindin-1 [OS=Homo sapiens]	12	4	53.8	5.25	1.724	0.931917
P04066	tissue alpha-L-fucosidase [OS=Homo sapiens]	8	3	53.7	6.84	1.722	0.931917
A0A0B4J1Y8	immunoglobulin lambda variable 9-49 [OS=Homo sapiens]	16	2	13	7.28	1.746	0.931917
P13797	Plastin-3 [OS=Homo sapiens]	4	2	70.8	5.6	1.718	0.931917
P10451-1	Osteopontin [OS=Homo sapiens]	49	12	35.4	4.58	1.724	0.931917
Q13509	tubulin beta-3 chain [OS=Homo sapiens]	7	2	50.4	4.93	1.717	0.931917
Q12860	Contactin-1 [OS=Homo sapiens]	6	4	113.2	5.9	0.669	0.936964
Q15828	Cystatin-M [OS=Homo sapiens]	21	2	16.5	8.09	0.668	0.936964
Q03154	Aminoacylase-1 [OS=Homo sapiens]	26	7	45.9	6.18	1.704	0.93757
P27169	Serum paraoxonase/arylesterase 1 [OS=Homo sapiens]	10	2	39.7	5.22	0.673	0.943888
A0A0C4DH68	immunoglobulin kappa variable 2-24 [OS=Homo sapiens]	33	2	13.1	8.53	0.674	0.944801
P98164	Low-density lipoprotein receptor-related protein 2 [OS=Homo sapiens]	16	57	521.6	5.08	1.649	0.947073
P01042-2	Isoform LMW of Kininogen-1 [OS=Homo sapiens]	56	22	47.9	6.65	0.675	0.947073
P35908	Keratin, type II cytoskeletal 2 epidermal [OS=Homo sapiens]	47	22	65.4	8	1.687	0.947073
P15309-1	Prostatic acid phosphatase [OS=Homo sapiens]	43	17	44.5	6.24	0.676	0.947073

P49221	protein-glutamine gamma-glutamyltransferase 4 [OS=Homo sapiens]	23	10	77.1	6.76	0.676	0.947073
P02753	Retinol-binding protein 4 [OS=Homo sapiens]	47	8	23	6.07	0.681	0.947073
P14384	Carboxypeptidase M [OS=Homo sapiens]	16	6	50.5	7.36	0.683	0.947073
P05362	Intercellular adhesion molecule 1 [OS=Homo sapiens]	16	6	57.8	7.99	1.649	0.947073
Q7Z5L0-1	Vitelline membrane outer layer protein 1 homolog [OS=Homo sapiens]	55	6	21.5	5.07	0.678	0.947073
O96009	Napsin-A [OS=Homo sapiens]	29	6	45.4	6.61	1.686	0.947073
P11279	Lysosome-associated membrane glycoprotein 1 [OS=Homo sapiens]	12	5	44.9	8.75	1.668	0.947073
P30086	phosphatidylethanolamine-binding protein 1 [OS=Homo sapiens]	49	5	21	7.53	1.631	0.947073
P29622	Kallistatin [OS=Homo sapiens]	12	5	48.5	7.75	0.695	0.947073
Q9H8L6	Multimerin-2 [OS=Homo sapiens]	4	4	104.3	5.86	1.633	0.947073
P23528	Cofilin-1 [OS=Homo sapiens]	32	3	18.5	8.09	1.63	0.947073
P15848	arylsulfatase B [OS=Homo sapiens]	9	3	59.6	8.21	1.652	0.947073
Q9NQ84-2	Isoform 2 of G-protein coupled receptor family C group 5 member C [OS=Homo sapiens]	6	2	49.4	8.43	0.686	0.947073
Q5VW32	BRO1 domain-containing protein BROX [OS=Homo sapiens]	8	2	46.4	7.65	0.694	0.947073
Q9BVM4	gamma-glutamylaminecyclotransferase [OS=Homo sapiens]	17	2	17.3	6.87	1.689	0.947073
P45877	peptidyl-prolyl cis-trans isomerase C [OS=Homo sapiens]	10	2	22.7	8.4	0.7	0.947073
P55290-4	Isoform 4 of Cadherin-13 [OS=Homo sapiens]	13	7	83.3	5.12	0.703	0.951903
P16278	Beta-galactosidase [OS=Homo sapiens]	24	12	76	6.57	1.622	0.953633
Q8NBJ4	Golgi membrane protein 1 [OS=Homo sapiens]	24	7	45.3	4.97	1.616	0.959848
P23083	Immunoglobulin heavy variable 1-2 [OS=Homo sapiens]	11	2	13.1	9.13	0.71	0.966978
P03973	Antileukoproteinase [OS=Homo sapiens]	20	2	14.3	8.75	1.609	0.967162
Q92820	Gamma-glutamyl hydrolase [OS=Homo sapiens]	39	9	35.9	7.11	1.605	0.970534
P21333	Filamin-A [OS=Homo sapiens]	2	2	280.6	6.06	1.604	0.970534
P00734	Prothrombin [OS=Homo sapiens]	33	16	70	5.9	1.598	0.977717
Q9H3G5	Probable serine carboxypeptidase CPVL [OS=Homo sapiens]	20	9	54.1	5.62	1.597	0.977717
Q07075	Glutamyl aminopeptidase [OS=Homo sapiens]	20	16	109.2	5.47	1.595	0.978879

O43707	Alpha-actinin-4 [OS=Homo sapiens]	11	7	104.8	5.44	1.594	0.978879
P54802	alpha-N-acetylglucosaminidase [OS=Homo sapiens]	44	23	82.2	6.65	0.718	0.979427
P35527	Keratin, type I cytoskeletal 9 [OS=Homo sapiens]	51	20	62	5.24	0.718	0.979427
P04217	Alpha-1B-glycoprotein [OS=Homo sapiens]	55	15	54.2	5.86	0.728	0.979427
P05090	apolipoprotein D [OS=Homo sapiens]	37	10	21.3	5.15	0.725	0.979427
P07602-3	Isoform Sap-mu-9 of Prosaposin [OS=Homo sapiens]	15	7	58.4	5.14	1.55	0.979427
P53634-1	Dipeptidyl peptidase 1 [OS=Homo sapiens]	22	7	51.8	6.99	1.552	0.979427
P19652	Alpha-1-acid glycoprotein 2 [OS=Homo sapiens]	48	9	23.6	5.11	0.718	0.979427
P09525	annexin A4 [OS=Homo sapiens]	25	6	35.9	6.13	1.546	0.979427
O95865	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2 [OS=Homo sapiens]	27	5	29.6	6.01	0.723	0.979427
P43251-2	Isoform 2 of Biotinidase [OS=Homo sapiens]	13	5	61.5	6.14	0.74	0.979427
P62937	peptidyl-prolyl cis-trans isomerase A [OS=Homo sapiens]	36	5	18	7.81	1.557	0.979427
P00915	carbonic anhydrase 1 [OS=Homo sapiens]	31	5	28.9	7.12	1.575	0.979427
P24821	Tenascin [OS=Homo sapiens]	3	4	240.7	4.89	1.579	0.979427
Q9H665	IGF-like family receptor 1 [OS=Homo sapiens]	16	4	37.9	7.08	1.585	0.979427
O75503	Ceroid-lipofuscinosis neuronal protein 5 [OS=Homo sapiens]	9	3	41.5	7.4	1.586	0.979427
Q6UXG3	CMRF35-like molecule 9 [OS=Homo sapiens]	11	3	36	5.92	0.732	0.979427
Q9UGN4-1	CMRF35-like molecule 8 [OS=Homo sapiens]	11	3	33.2	5.49	1.571	0.979427
Q14508	WAP four-disulfide core domain protein 2 [OS=Homo sapiens]	27	3	13	4.84	1.547	0.979427
P23526-1	Adenosylhomocysteinase [OS=Homo sapiens]	9	3	47.7	6.34	1.568	0.979427
P45880-1	Isoform 1 of Voltage-dependent anion-selective channel protein 2 [OS=Homo sapiens]	12	3	33.4	7.59	0.735	0.979427
Q8IUL8	Cartilage intermediate layer protein 2 [OS=Homo sapiens]	3	2	126.2	8.22	0.736	0.979427
Q16651	prostasin [OS=Homo sapiens]	10	2	36.4	5.85	0.74	0.979427
P35052	Glypican-1 [OS=Homo sapiens]	6	2	61.6	7.3	1.564	0.979427
Q9NU53	Glycoprotein integral membrane protein 1 [OS=Homo sapiens]	9	2	36.8	4.91	0.731	0.979427
P28799	Granulins [OS=Homo sapiens]	8	2	63.5	6.83	1.587	0.979427

P00739-2	Isoform 2 of Haptoglobin-related protein [OS=Homo sapiens]	27	11	43	7.08	1.573	0.979427
P20138-1	Myeloid cell surface antigen CD33 [OS=Homo sapiens]	8	2	39.8	8.38	0.738	0.979427
P62805	histone H4 [OS=Homo sapiens]	41	4	11.4	11.36	0.742	0.983015
P02768-1	Serum albumin [OS=Homo sapiens]	74	52	69.3	6.28	0.901	0.983586
O60494	cubilin [OS=Homo sapiens]	23	52	398.5	5.35	1.21	0.983586
P02787	Serotransferrin [OS=Homo sapiens]	72	45	77	7.12	1.514	0.983586
P15144	aminopeptidase N [OS=Homo sapiens]	40	34	109.5	5.48	1.348	0.983586
P00450	Ceruloplasmin [OS=Homo sapiens]	40	33	122.1	5.72	0.879	0.983586
P01133-1	Pro-epidermal growth factor [OS=Homo sapiens]	28	31	133.9	5.85	0.997	0.983586
O43451	Maltase-glucoamylase, intestinal [OS=Homo sapiens]	20	27	209.7	5.5	1.257	0.983586
P07911-5	Isoform 5 of Uromodulin [OS=Homo sapiens]	50	26	73.5	5.26	0.768	0.983586
P02751	fibronectin [OS=Homo sapiens]	17	24	262.5	5.71	0.867	0.983586
P01833	Polymeric immunoglobulin receptor [OS=Homo sapiens]	42	24	83.2	5.74	0.883	0.983586
P04264	Keratin, type II cytoskeletal 1 [OS=Homo sapiens]	45	26	66	8.12	0.823	0.983586
P13645	Keratin, type I cytoskeletal 10 [OS=Homo sapiens]	49	26	58.8	5.21	1.175	0.983586
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein [OS=Homo sapiens]	8	22	468.5	6.51	0.836	0.983586
Q9BXP8-1	Pappalysin-2 [OS=Homo sapiens]	19	21	198.4	5.47	1.396	0.983586
P02774-3	Isoform 3 of Vitamin D-binding protein [OS=Homo sapiens]	47	18	55.1	5.74	1.254	0.983586
P12109	Collagen alpha-1(VI) chain [OS=Homo sapiens]	27	17	108.5	5.43	0.972	0.983586
P01011-1	Alpha-1-antichymotrypsin [OS=Homo sapiens]	49	17	47.6	5.52	0.97	0.983586
P01023	alpha-2-macroglobulin [OS=Homo sapiens]	17	17	163.2	6.46	0.884	0.983586
P00747	Plasminogen [OS=Homo sapiens]	34	17	90.5	7.24	0.832	0.983586
Q12805	EGF-containing fibulin-like extracellular matrix protein 1 [OS=Homo sapiens]	49	16	54.6	5.07	1.31	0.983586
P08473	Neprilysin [OS=Homo sapiens]	28	16	85.5	5.73	1.352	0.983586
P02647	Apolipoprotein A-I [OS=Homo sapiens]	52	16	30.8	5.76	0.947	0.983586
P02671-1	Fibrinogen alpha chain [OS=Homo sapiens]	28	15	94.9	6.01	0.969	0.983586

P05155-3	Isoform 3 of Plasma protease C1 inhibitor [OS=Homo sapiens]	31	15	55.7	6.4	0.927	0.983586
P19320-1	Vascular cell adhesion protein 1 [OS=Homo sapiens]	27	15	81.2	5.22	0.87	0.983586
Q12907	Vesicular integral-membrane protein VIP36 [OS=Homo sapiens]	42	14	40.2	6.95	0.806	0.983586
O00391	Sulfhydryl oxidase 1 [OS=Homo sapiens]	25	14	82.5	8.92	0.955	0.983586
P22891-2	Isoform 2 of Vitamin K-dependent protein Z [OS=Homo sapiens]	36	14	47	6.04	1.363	0.983586
P02679	Fibrinogen gamma chain [OS=Homo sapiens]	43	12	51.5	5.62	1.46	0.983586
P07355-2	Isoform 2 of Annexin A2 [OS=Homo sapiens]	43	12	40.4	8.37	0.935	0.983586
P22792	Carboxypeptidase n subunit 2 [OS=Homo sapiens]	33	12	60.5	5.99	1.2	0.983586
Q16270-1	Insulin-like growth factor-binding protein 7 [OS=Homo sapiens]	48	12	29.1	7.9	1.196	0.983586
P05154	Plasma serine protease inhibitor [OS=Homo sapiens]	37	12	45.6	9.26	0.793	0.983586
O95336	6-phosphogluconolactonase [OS=Homo sapiens]	67	11	27.5	6.05	0.858	0.983586
P11117	Lysosomal acid phosphatase [OS=Homo sapiens]	26	11	48.3	6.74	1.009	0.983586
P15586	N-acetylglucosamine-6-sulfatase [OS=Homo sapiens]	23	10	62	8.31	1.252	0.983586
P14543-1	Nidogen-1 [OS=Homo sapiens]	15	10	136.3	5.29	0.971	0.983586
P0DOX5	immunoglobulin gamma-1 heavy chain [OS=Homo sapiens]	48	18	49.3	8.72	1.537	0.983586
P02649	Apolipoprotein E [OS=Homo sapiens]	37	10	36.1	5.73	0.943	0.983586
Q8WZ75-1	Roundabout homolog 4 [OS=Homo sapiens]	12	10	107.4	6.64	1.165	0.983586
P02750	Leucine-rich alpha-2-glycoprotein [OS=Homo sapiens]	45	10	38.2	6.95	0.893	0.983586
P0DOX2	Immunoglobulin alpha-2 heavy chain [OS=Homo sapiens]	49	15	48.9	6.81	0.889	0.983586
P01876	immunoglobulin heavy constant alpha 1 [OS=Homo sapiens]	63	13	37.6	6.51	0.886	0.983586
P02749	Beta-2-glycoprotein 1 [OS=Homo sapiens]	40	9	38.3	7.97	0.784	0.983586
P19013	Keratin, type II cytoskeletal 4 [OS=Homo sapiens]	29	10	57.3	6.61	1.246	0.983586
P02538	Keratin, type II cytoskeletal 6A [OS=Homo sapiens]	28	13	60	8	1.275	0.983586
Q9BYE9	Cadherin-related family member 2 [OS=Homo sapiens]	9	9	141.5	4.5	1.166	0.983586
P12830	Cadherin-1 [OS=Homo sapiens]	16	9	97.4	4.73	0.793	0.983586
P19022	Cadherin-2 [OS=Homo sapiens]	16	9	99.7	4.81	0.951	0.983586
P16444	dipeptidase 1 [OS=Homo sapiens]	34	9	45.6	6.15	1.49	0.983586

P05156	Complement factor I [OS=Homo sapiens]	21	9	65.7	7.5	1.389	0.983586
P60174	Triosephosphate isomerase [OS=Homo sapiens]	44	8	30.8	5.92	1.392	0.983586
Q9HAT2	sialate O-acetylesterase [OS=Homo sapiens]	19	8	58.3	7.33	1.341	0.983586
P06733-1	alpha-enolase [OS=Homo sapiens]	31	10	47.1	7.39	1.334	0.983586
P02763	Alpha-1-acid glycoprotein 1 [OS=Homo sapiens]	49	10	23.5	5.02	1.134	0.983586
P43652	Afamin [OS=Homo sapiens]	19	8	69	5.9	1.24	0.983586
P27487	Dipeptidyl peptidase 4 [OS=Homo sapiens]	12	8	88.2	6.04	0.988	0.983586
P13646	Keratin, type I cytoskeletal 13 [OS=Homo sapiens]	33	15	49.6	4.96	0.908	0.983586
P01859	Immunoglobulin heavy constant gamma 2 [OS=Homo sapiens]	62	14	35.9	7.59	0.956	0.983586
P01871-2	Isoform 2 of Immunoglobulin heavy constant mu [OS=Homo sapiens]	21	8	51.9	6.15	1.198	0.983586
P19835-1	Bile salt-activated lipase [OS=Homo sapiens]	16	8	79.3	5.34	1.015	0.983586
P08174-7	Isoform 7 of Complement decay-accelerating factor [OS=Homo sapiens]	22	8	59	8.78	0.998	0.983586
Q01459	Di-N-acetylchitobiase [OS=Homo sapiens]	28	7	43.7	6.64	0.91	0.983586
Q96NY8	Nectin-4 [OS=Homo sapiens]	18	7	55.4	5.38	0.802	0.983586
P68871	Hemoglobin subunit beta [OS=Homo sapiens]	90	12	16	7.28	0.766	0.983586
Q8NCC3	Group XV phospholipase A2 [OS=Homo sapiens]	22	7	46.6	6.73	1.207	0.983586
Q9NZP8	Complement C1r subcomponent-like protein [OS=Homo sapiens]	20	7	53.5	7.2	0.751	0.983586
O00560-1	Syntenin-1 [OS=Homo sapiens]	40	7	32.4	7.53	1.356	0.983586
P13647	keratin, type II cytoskeletal 5 [OS=Homo sapiens]	22	11	62.3	7.74	1.146	0.983586
P15289-2	Isoform 2 of Arylsulfatase A [OS=Homo sapiens]	28	7	44.9	5.87	1.183	0.983586
P50895	Basal cell adhesion molecule [OS=Homo sapiens]	15	7	67.4	5.81	1.009	0.983586
Q8NFZ8	Cell adhesion molecule 4 [OS=Homo sapiens]	26	7	42.8	6.3	0.973	0.983586
P18065	Insulin-like growth factor-binding protein 2 [OS=Homo sapiens]	31	7	34.8	7.5	1.444	0.983586
P43121	Cell surface glycoprotein MUC18 [OS=Homo sapiens]	19	7	71.6	5.76	1.003	0.983586
P15311	Ezrin [OS=Homo sapiens]	27	13	69.4	6.27	1.372	0.983586
P00558	phosphoglycerate kinase 1 [OS=Homo sapiens]	26	7	44.6	8.1	1.349	0.983586
Q99715-1	Collagen alpha-1(XII) chain [OS=Homo sapiens]	4	7	332.9	5.53	1.244	0.983586

O14498	Immunoglobulin superfamily containing leucine-rich repeat protein [OS=Homo sapiens]	23	7	46	5.15	0.756	0.983586
P08294	Extracellular superoxide dismutase [Cu-Zn] [OS=Homo sapiens]	37	7	25.8	6.61	0.814	0.983586
P11142-1	Heat shock cognate 71 kDa protein [OS=Homo sapiens]	18	8	70.9	5.52	1.502	0.983586
P07195	L-lactate dehydrogenase B chain [OS=Homo sapiens]	25	8	36.6	6.05	1.353	0.983586
P06280	alpha-galactosidase A [OS=Homo sapiens]	20	7	48.7	5.6	1.139	0.983586
Q96RW7-1	Hemicentin-1 [OS=Homo sapiens]	2	7	613	6.49	0.784	0.983586
P16870	Carboxypeptidase E [OS=Homo sapiens]	26	7	53.1	5.14	0.858	0.983586
Q99519	sialidase-1 [OS=Homo sapiens]	17	6	45.4	5.88	1.203	0.983586
P04406	glyceraldehyde-3-phosphate dehydrogenase [OS=Homo sapiens]	28	6	36	8.46	1.436	0.983586
P15941-2	Isoform 2 of Mucin-1 [OS=Homo sapiens]	6	6	122.9	7.56	1.235	0.983586
P26992	Ciliary neurotrophic factor receptor subunit alpha [OS=Homo sapiens]	30	6	40.6	6.76	0.841	0.983586
P01034	Cystatin-C [OS=Homo sapiens]	45	6	15.8	8.75	1.275	0.983586
P01019	Angiotensinogen [OS=Homo sapiens]	20	6	53.1	6.32	1.215	0.983586
P30530	Tyrosine-protein kinase receptor UFO [OS=Homo sapiens]	11	6	98.3	5.43	1.166	0.983586
P17900	Ganglioside GM2 activator [OS=Homo sapiens]	41	6	20.8	5.31	1.196	0.983586
P08779	Keratin, type I cytoskeletal 16 [OS=Homo sapiens]	33	12	51.2	5.05	0.922	0.983586
P00352	Retinal dehydrogenase 1 [OS=Homo sapiens]	17	6	54..8	6.73	1.463	0.983586
P04004	Vitronectin [OS=Homo sapiens]	15	6	54.3	5.8	1.155	0.983586
Q8IV08	Phospholipase D3 [OS=Homo sapiens]	16	6	54.7	6.47	0.871	0.983586
Q02487	Desmocollin-2 [OS=Homo sapiens]	11	6	99.9	5.34	1.127	0.983586
Q96DA0	Zymogen granule protein 16 homolog B [OS=Homo sapiens]	43	6	22.7	7.39	0.842	0.983586
P06727	Apolipoprotein A-IV [OS=Homo sapiens]	18	6	45.4	5.38	0.931	0.983586
O75594	peptidoglycan recognition protein 1 [OS=Homo sapiens]	53	6	21.7	8.59	0.961	0.983586
P06870-1	Kallikrein-1 [OS=Homo sapiens]	54	6	28.9	4.83	0.911	0.983586
Q9Y2E5	Epididymis-specific alpha-mannosidase [OS=Homo sapiens]	8	6	11.9	7.24	1.266	0.983586
P19440	glutathione hydrolase 1 proenzyme [OS=Homo sapiens]	14	6	61.4	7.12	1.468	0.983586

Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1 [OS=Homo sapiens]	20	6	35.2	8.28	1.148	0.983586
P54760	Ephrin type-B receptor 4 [OS=Homo sapiens]	9	6	108.2	6.9	0.924	0.983586
Q12913-1	Receptor-type tyrosine-protein phosphatase eta [OS=Homo sapiens]	8	6	145.9	5.58	0.917	0.983586
P09603	Macrophage colony-stimulating factor 1 [OS=Homo sapiens]	14	6	60.1	5.29	1.362	0.983586
Q8IYS5-7	Isoform 7 of Osteoclast-associated immunoglobulin-like receptor [OS=Homo sapiens]	30	5	30.9	6.52	1.294	0.983586
P16152	Carbonyl reductase [NADPH] 1 [OS=Homo sapiens]	25	5	30.4	8.32	1.233	0.983586
Q9UNN8	Endothelial protein C receptor [OS=Homo sapiens]	26	5	26.7	7.18	0.929	0,983586
Q96IU4	Protein ABHD14B [OS=Homo sapiens]	33	5	22.3	6.4	0.973	0.983586
P42785-2	Isoform 2 of Lysosomal Pro-X carboxypeptidase [OS=Homo sapiens]	15	5	58.1	7.4	1..013	0.983586
P30041	Peroxiredoxin-6 [OS=Homo sapiens]	29	5	25	6.38	1.368	0.983586
O00241	Signal-regulatory protein beta-1 [OS=Homo sapiens]	17	5	43.2	6.52	0.935	0.983586
P10619	lysosomal protective protein [OS=Homo sapiens]	12	5	54.4	6.61	1.441	0.983586
P22105-4	Isoform 5 of Tenascin-X [OS=Homo sapiens]	2	5	458.2	5.17	1.527	0.983586
P08236	beta-glucuronidase [OS=Homo sapiens]	9	5	74.7	7.02	1.239	0.983586
Q9H6S3	epidermal growth factor receptor kinase substrate 8-like protein 2 [OS=Homo sapiens]	10	5	80.6	6.84	0.899	0.983586
Q9Y646	Carboxypeptidase Q [OS=Homo sapiens]	15	5	51.9	6.18	1.452	0.983586
P08138-2	Isoform 2 of Tumor necrosis factor receptor superfamily member 16 [OS=Homo sapiens]	26	5	35.7	4.7	1.327	0.983586
Q8NHP8	Putative phospholipase B-like 2 [OS=Homo sapiens]	11	5	65.4	6.8	1.193	0.983586
P36955	Pigment epithelium-derived factor [OS=Homo sapiens]	15	5	46.3	6.38	0.983	0.983586
P33908	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA [OS=Homo sapiens]	11	5	72.9	6.47	0.934	0.983586
P00918	Carbonic anhydrase 2 [OS=Homo sapiens]	25	5	29.2	7.4	1.279	0.983586
P14618	Pyruvate kinase PKM [OS=Homo sapiens]	16	5	57.9	7.84	1.268	0.983586
P25705-1	ATP synthase subunit alpha, mitochondrial [OS=Homo sapiens]	12	5	59.7	9.13	1.286	0.983586
P04745	alpha-amylase 1 [OS=Homo sapiens]	60	20	57.7	6.93	0.801	0.983586
P68104	Elongation factor 1-alpha 1 [OS=Homo sapiens]	15	5	50.1	9.01	1.214	0.983586

Q03403	Trefoil factor 2 [OS=Homo sapiens]	51	5	14.3	5.81	0.789	0.983586
O75874	Isocitrate dehydrogenase [NADP] cytoplasmic [OS=Homo sapiens]	16	6	46.6	7.01	1.414	0.983586
P01591	Immunoglobulin J chain [OS=Homo sapiens]	37	5	18.1	5.24	0.802	0.983586
P22352	Glutathione peroxidase 3 [OS=Homo sapiens]	28	5	25.5	8.13	0.894	0.983586
P08758	annexin A5 [OS=Homo sapiens]	16	5	35.9	5.05	1.002	0.983586
Q86UN3	Reticulon-4 receptor-like 2 [OS=Homo sapiens]	14	5	46.1	7.62	0.91	0.983586
P61970	nuclear transport factor 2 [OS=Homo sapiens]	69	5	14.5	5.38	1.464	0.983586
Q86T13	C-type lectin domain family 14 member A [OS=Homo sapiens]	18	5	51.6	6.35	0.896	0.983586
Q08174-2	Isoform 2 of Protocadherin-1 [OS=Homo sapiens]	6	5	133.6	5.06	1.016	0.983586
Q8WUM4-2	Isoform 2 of Programmed cell death 6-interacting protein [OS=Homo sapiens]	9	5	96.7	6.52	1.225	0.983586
P06576	ATP synthase subunit beta, mitochondrial [OS=Homo sapiens]	13	5	56.5	5.4	1.411	0.983586
Q96AP7	Endothelial cell-selective adhesion molecule [OS=Homo sapiens]	15	4	41.2	9.32	0.817	0.983586
O00462	Beta-mannosidase [OS=Homo sapiens]	5	4	100.8	5.52	1.376	0.983586
O43505	beta-1,4-glucuronyltransferase 1 [OS=Homo sapiens]	12	4	47.1	7.2	0.985	0.983586
Q9BY67-3	Isoform 3 of Cell adhesion molecule 1 [OS=Homo sapiens]	18	4	51.5	4.87	1.016	0.983586
P53990-5	Isoform 5 of IST1 homolog [OS=Homo sapiens]	14	4	41.5	5.57	1.185	0.983586
P17050	alpha-N-acetylgalactosaminidase [OS=Homo sapiens]	11	4	46.5	5.19	1.466	0.983586
O95967	EGF-containing fibulin-like extracellular matrix protein 2 [OS=Homo sapiens]	14	4	49.4	4.94	0.75	0.983586
P63104	14-3-3 protein zeta/delta [OS=Homo sapiens]	26	5	27.7	4.79	1.477	0.983586
P13987	CD59 glycoprotein [OS=Homo sapiens]	20	4	14.2	6.48	0.854	0.983586
P15328	Folate receptor alpha [OS=Homo sapiens]	18	4	29.8	7.97	1.244	0.983586
Q06830	peroxiredoxin-1 [OS=Homo sapiens]	31	5	22.1	8.13	1.421	0.983586
Q13421-2	Isoform 3 of Mesothelin [OS=Homo sapiens]	8	4	71.4	7.36	1.181	0.983586
O75340	programmed cell death protein 6 [OS=Homo sapiens]	35	4	21.9	5.4	1.309	0.983586
P10153	Non-secretory ribonuclease [OS=Homo sapiens]	22	4	18.3	8.73	0.913	0.983586
Q6UVK1	Chondroitin sulfate proteoglycan 4 [OS=Homo sapiens]	2	4	250.4	5.47	1.192	0.983586

Q9ULI3	Protein HEG homolog 1 [OS=Homo sapiens]	6	4	147.4	6.18	1.52	0.983586
P30740	Leukocyte elastase inhibitor [OS=Homo sapiens]	13	4	42.7	6.28	1.14	0.983586
P09211	Glutathione S-transferase P [OS=Homo sapiens]	30	4	23.3	5.64	0.39	0.983586
P12111	Collagen alpha-3(VI) chain [OS=Homo sapiens]	2	4	343.5	6.68	1.425	0.983586
P32119	Peroxiredoxin-2 [OS=Homo sapiens]	23	5	21.9	5.97	1.448	0.983586
P23142-4	Isoform C of Fibulin-1 [OS=Homo sapiens]	9	4	74.4	5.24	0.816	0.983586
P39060-3	Collagen alpha-1(XVIII) chain [OS=Homo sapiens]	5	4	178.1	6.01	0.819	0.983586
P13727	Bone marrow proteoglycan [OS=Homo sapiens]	25	4	25.2	6.76	1.465	0.983586
P15291	Beta-1,4-galactosyltransferase 1 [OS=Homo sapiens]	19	4	43.9	8.65	1.167	0.983586
P08697-1	Alpha-2-antiplasmin [OS=Homo sapiens]	15	4	54.5	6.29	1.286	0.983586
P56537	eukaryotic translation initiation factor 6 [OS=Homo sapiens]	33	4	26.6	4.68	1.392	0.983586
P16070	CD44 antigen [OS=Homo sapiens]	7	4	81.5	5.33	0.987	0.983586
P15814	Immunoglobulin lambda-like polypeptide 1 [OS=Homo sapiens]	23	3	22.9	10.07	1.243	0.983586
P05937	Calbindin [OS=Homo sapiens]	13	3	30	4.83	1.001	0.983586
P15151-1	Poliovirus receptor [OS=Homo sapiens]	10	3	45.3	6.52	1.193	0.983586
O75144-2	Isoform 2 of ICOS ligand [OS=Homo sapiens]	14	3	34.2	5.11	1.205	0.983586
P16112	Aggrecan core protein [OS=Homo sapiens]	1	3	250	4.18	0.987	0.983586
P78324-2	Isoform 2 of Tyrosine-protein phosphatase non-receptor type substrate 1 [OS=Homo sapiens]	28	8	55.4	6.98	0.875	0.983586
P11021	78 kDa glucose-regulated protein [OS=Homo sapiens]	7	3	72.3	5.16	0.93	0.983586
P01040	Cystatin-A [OS=Homo sapiens]	53	3	11	5.5	0.753	0.983586
Q9UGT4	Sushi domain-containing protein 2 [OS=Homo sapiens]	5	3	90.2	6.28	0.759	0.983586
P17931	Galectin-3 [OS=Homo sapiens]	15	3	26.1	8.56	1.25	0.983586
P04433	Immunoglobulin kappa variable 3-11 [OS=Homo sapiens]	52	3	12.6	4.96	0.816	0.983586
Q07507	Dermatopontin [OS=Homo sapiens]	18	3	24	4.82	1.152	0.983586
P01619	Immunoglobulin kappa variable 3-20 [OS=Homo sapiens]	47	5	12.5	4.96	0.884	0.983586
Q14982-4	Isoform 4 of Opioid-binding protein/cell adhesion molecule [OS=Homo sapiens]	14	3	38.8	6.55	1.333	0.983586

P98172	ephrin-B1 [OS=Homo sapiens]	16	3	38	8.94	1.32	0.983586
P40189-1	Interleukin-6 receptor subunit beta [OS=Homo sapiens]	4	3	103.5	5.95	0.978	0.983586
P01860	Immunoglobulin heavy constant gamma 3 [OS=Homo sapiens]	35	11	41.3	7.9	1.425	0.983586
Q96FE7-1	Phosphoinositide-3-kinase-interacting protein 1 [OS=Homo sapiens]	14	3	28.2	5.01	1.185	0.983586
Q6P531	glutathione hydrolase 6 [OS=Homo sapiens]	11	3	50.5	6.07	0.796	0.983586
Q13228-4	Isoform 4 of Selenium-binding protein 1 [OS=Homo sapiens]	8	3	56.8	6.48	1.459	0.983586
Q9P121-4	Isoform 4 of Neurotrimin [OS=Homo sapiens]	12	3	39.2	8	1.333	0.983586
P23284	peptidyl-prolyl cis-trans isomerase B [OS=Homo sapiens]	16	3	23.7	9.41	0.761	0.983586
P61916	Epididymal secretory protein E1 [OS=Homo sapiens]	32	3	16.6	7.65	0.841	0.983586
P0C0L5	Complement C4-B [OS=Homo sapiens]	33	40	192.6	7.27	0.799	0.983586
P61026	ras-related protein rab-10 [OS=Homo sapiens]	17	3	22.5	8.38	1.236	0.983586
Q7Z3B1	Neuronal growth regulator 1 [OS=Homo sapiens]	14	3	38.7	6.21	0.896	0.983586
Q08345-5	Isoform 4 of Epithelial discoidin domain-containing receptor 1 [OS=Homo sapiens]	4	3	101.7	6.83	0.857	0.983586
Q496F6	CMRF35-like molecule 2 [OS=Homo sapiens]	18	3	22.9	8	1.371	0.983586
Q9UIB8	SLAM family member 5 [OS=Homo sapiens]	15	3	38.8	7.06	1.422	0.983586
P31997	carcinoembryonic antigen-related cell adhesion molecule 8 [OS=Homo sapiens]	12	3	38.1	7.39	1.134	0.983586
P06865	Beta-hexosaminidase subunit alpha [OS=Homo sapiens]	8	4	60.7	5.16	1.39	0.983586
Q14515	SPARC-like protein 1 [OS=Homo sapiens]	6	3	75.2	4.81	0.902	0.983586
P50897	Palmitoyl-protein thioesterase 1 [OS=Homo sapiens]	16	3	34.2	6.52	1.4	0.983586
Q969P0	Immunoglobulin superfamily member 8 [OS=Homo sapiens]	7	3	65	8	1.467	0.983586
Q9HB40	Retinoid-inducible serine carboxypeptidase [OS=Homo sapiens]	8	3	50.8	5.81	1.469	0.983586
P19827-1	Inter-alpha-trypsin inhibitor heavy chain H1 [OS=Homo sapiens]	6	3	101.3	6.79	1.391	0.983586
Q9UBX5	Fibulin-5 [OS=Homo sapiens]	7	3	50.1	4.73	0.905	0.983586
P40925-3	Isoform 3 of Malate dehydrogenase, cytoplasmic [OS=Homo sapiens]	11	3	38.6	7.71	1.318	0.983586
P22223-1	Cadherin-3 [OS=Homo sapiens]	5	3	91.4	4.75	1.523	0.983586
O00584	Ribonuclease T2 [OS=Homo sapiens]	12	3	29.5	7.08	0.772	0.983586

P0DOX8	Immunoglobulin lambda-1 light chain [OS=Homo sapiens]	37	6	22.8	6.76	0.826	0.983586
P13591-2	Neural cell adhesion molecule 1 [OS=Homo sapiens]	4	2	94.5	4.87	0.941	0.983586
P31431-1	syndecan-4 [OS=Homo sapiens]	14	2	21.6	4.5	1.174	0.983586
P19801-2	Isoform 2 of Amiloride-sensitive amine oxidase [copper-containing] [OS=Homo sapiens]	4	2	87.2	7.17	0.899	0.983586
Q9UBR2	Cathepsin Z [OS=Homo sapiens]	9	2	33.8	7.11	0.828	0.983586
P07737	profilin-1 [OS=Homo sapiens]	19	2	15	8.27	1.203	0.983586
P55000	Secreted Ly-6/uPAR-related protein 1 [OS=Homo sapiens]	52	2	11.2	5.33	1.202	0.983586
P29972-1	aquaporin-1 [OS=Homo sapiens]	13	2	28.5	7.42	0.898	0.983586
P04899-4	Isoform sGi2 of Guanine nucleotide-binding protein G(i) subunit alpha-2 [OS=Homo sapiens]	7	2	41.5	5.99	0.848	0.983586
P12821-1	Angiotensin-converting enzyme [OS=Homo sapiens]	2	2	149.6	6.39	1.507	0.983586
Q9UN70-1	Protocadherin gamma-C3 [OS=Homo sapiens]	2	2	101	5.21	1.012	0.983586
Q9GZX9	Twisted gastrulation protein homolog 1 [OS=Homo sapiens]	16	2	25	5.34	0.923	0.983586
Q00796	Sorbitol dehydrogenase [OS=Homo sapiens]	10	2	38.3	7.97	1.33	0.983586
Q96J84-2	Isoform 2 of Kin of IRRE-like protein 1 [OS=Homo sapiens]	4	2	85	5.73	1.155	0.983586
Q8TDQ1-6	Isoform 6 of CMRF35-like molecule 1 [OS=Homo sapiens]	8	2	32.6	5.99	0.996	0.983586
O15393-2	Isoform 2 of Transmembrane protease serine 2 [OS=Homo sapiens]	6	2	57.7	5.44	0.793	0.983586
Q04695	Keratin, type I cytoskeletal 17 [OS=Homo sapiens]	15	7	48.1	5.02	1.136	0.983586
A0A0A0MS15	immunoglobulin heavy variable 3-49 [OS=Homo sapiens]	17	2	13	8.62	1.303	0.983586
Q8N6Q3	CD177 antigen [OS=Homo sapiens]	10	2	46.3	6.29	1.257	0.983586
P07359	Platelet glycoprotein Ib alpha chain [OS=Homo sapiens]	3	2	71.5	6.29	0.817	0.983586
A6NI73-1	Leukocyte immunoglobulin-like receptor subfamily A member 5 [OS=Homo sapiens]	15	2	32.7	6.99	1.372	0.983586
Q01469	Fatty acid-binding protein, epidermal [OS=Homo sapiens]	13	2	15.2	7.01	0.874	0.983586
Q8TF66-2	Isoform 2 of Leucine-rich repeat-containing protein 15 [OS=Homo sapiens]	4	2	65	6.71	0.755	0.983586
P00748	Coagulation factor XII [OS=Homo sapiens]	6	2	67.7	7.74	1.364	0.983586
P06312	immunoglobulin kappa variable 4-1 [OS=Homo sapiens]	20	2	13.4	5.25	0.841	0.983586

P13533	myosin-6 [OS=Homo sapiens]	3	5	223.6	5.73	0.781	0.983586
A0A0C4DH25	immunoglobulin kappa variable 3D-20 [OS=Homo sapiens]	28	4	12.5	4.59	0.823	0.983586
Q9UM22	Mammalian ependymin-related protein 1 [OS=Homo sapiens]	10	2	25.4	6.6	0.963	0.983586
P0DOX7	immunoglobulin kappa light chain [OS=Homo sapiens]	53	10	23.4	7.17	0.993	0.983586
P01599	Immunoglobulin kappa variable 1-17 [OS=Homo sapiens]	23	2	12.8	8.68	0.826	0.983586
P01614	Immunoglobulin kappa variable 2D-40 [OS=Homo sapiens]	28	3	13.3	4.61	0951	0.983586
P61224-1	Ras-related protein Rap-1b [OS=Homo sapiens]	13	2	20.8	5.78	1.364	0.983586
O43278	Kunitz-type protease inhibitor 1 [OS=Homo sapiens]	4	2	58.4	6.29	0.91	0.983586
P14174	Macrophage Migration inhibitory factor [OS=Homo sapiens]	17	2	12.5	7.88	1.262	0.983586
O00592	Podocalyxin [OS=Homo sapiens]	4	2	58.6	5.49	1.188	0.983586
P18669	Phosphoglycerate mutase 1 [OS=Homo sapiens]	14	2	28.8	7.18	1.395	0.983586
P07996	thrombospondin-1 [OS=Homo sapiens]	2	2	129.3	4.94	1.349	0.983586
P31025	Lipocalin-1 [OS=Homo sapiens]	13	2	19.2	5.58	1.222	0.983586
Q8N4F0	BPI fold-containing family B member 2 [OS=Homo sapiens]	7	2	49.1	8.72	1.381	0.983586
P07998	Ribonuclease pancreatic [OS=Homo sapiens]	30	2	17.6	8.79	1.124	0.983586
P11684	Uteroglobin [OS=Homo sapiens]	42	2	10	5.06	0.799	0.983586
P63000-2	Isoform B of Ras-related C3 botulinum toxin substrate 1 [OS=Homo sapiens]	9	2	23.5	8.63	1.389	0.983586
Q6UX71-1	Plexin domain-containing protein 2 [OS=Homo sapiens]	5	2	59.5	6.46	1.184	0.983586
P08727	Keratin, type I cytoskeletal 19 [OS=Homo sapiens]	16	6	44.1	5.14	0.865	0.983586
Q99574	Neuroserpin [OS=Homo sapiens]	6	2	46.4	4.91	0.789	0.983586
Q9UMR5-3	Isoform 3 of Lysosomal thioesterase PPT2 [OS=Homo sapiens]	10	2	34.8	6.46	1.284	0.983586
P36957	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial [OS=Homo sapiens]	4	2	48.7	8.95	0.863	0.983586
Q14393-1	Growth arrest-specific protein 6 [OS=Homo sapiens]	5	2	79.6	6.21	1.352	0.983586
P04430	Immunoglobulin kappa variable 1-16 [OS=Homo sapiens]	29	2	12.6	8.16	0.958	0.983586
P21266	glutathione S-transferase Mu 3 [OS=Homo sapiens]	11	2	26.5	5.54	0.852	0.983586

Q5T2W1-1	Na(+)/H(+) exchange regulatory cofactor NHE-RF3 [OS=Homo sapiens]	6	2	57.1	5.55	1.129	0.983586
A0A0B4J1X5	immunoglobulin heavy variable 3-74 [OS=Homo sapiens]	25	4	12.8	8.66	0.908	0.983586
P05413	Fatty acid-binding protein, heart [OS=Homo sapiens]	17	2	14.8	6.8	1.186	0.983586
P41181	aquaporin-2 [OS=Homo sapiens]	8	2	28.8	6.93	1.298	0.983586
P01594	Immunoglobulin kappa variable 1-33 [OS=Homo sapiens]	34	2	12.8	4.78	0.947	0.983586
A0A0B4J2D9	immunoglobulin kappa variable 1D-13 [OS=Homo sapiens]	29	2	12.6	7.84	0.765	0.983586
P34059	N-acetylgalactosamine-6-sulfatase [OS=Homo sapiens]	8	2	58	6.74	1.249	0.983586
A0A075B6S5	immunoglobulin kappa variable 1-27 [OS=Homo sapiens]	54	3	12.7	8.29	1.2	0.983586
Q9BRT3	migration and invasion enhancer 1 [OS=Homo sapiens]	30	2	12.4	4.37	1.213	0.983586
O43633	charged multivesicular body protein 2a [OS=Homo sapiens]	9	2	25.1	5.97	0.888	0.983586
P01701	immunoglobulin lambda variable 1-51 [OS=Homo sapiens]	32	2	12.2	7.03	1.242	0.983586
P55285-1	Cadherin-6 [OS=Homo sapiens]	3	2	88.3	4.93	1.324	0.983586
Q9HD89	resistin [OS=Homo sapiens]	24	2	11.4	6.86	1.371	0.983586
A0A0C4DH38	immunoglobulin heavy variable 5-51 [OS=Homo sapiens]	38	3	12.7	8.27	1.007	0.983586
P05026	Sodium/potassium-transporting ATPase subunit beta-1 [OS=Homo sapiens]	9	2	35	8.53	1.512	0.983586
P61626	lysozyme c [OS=Homo sapiens]	27	2	16.5	9.16	1.018	0.983917
P00738	Haptoglobin [OS=Homo sapiens]	57	20	45.2	6.58	1.122	0.984832
Q14624-1	Inter-alpha-trypsin inhibitor heavy chain H4 [OS=Homo sapiens]	28	25	10.3.3	6.98	1.12	0.985411
P01033	Metalloproteinase inhibitor 1 [OS=Homo sapiens]	20	3	23.2	8.1	1.021	0.986584
O14773	Tripeptidyl-peptidase 1 [OS=Homo sapiens]	19	6	61.2	6.48	1.031	0.992101
P08572	Collagen alpha-2(IV) chain [OS=Homo sapiens]	2	3	167.4	8.66	1.111	0.992101
P01834	immunoglobulin kappa constant [OS=Homo sapiens]	90	10	11.8	6.52	1.031	0.992101
P04432	Immunoglobulin kappa variable 1D-39 [OS=Homo sapiens]	29	2	12.7	8.66	1.029	0.992101
P09619	Platelet-derived growth factor receptor beta [OS=Homo sapiens]	2	2	123.9	4.98	1.026	0.992101
P00751-1	Complement factor B [OS=Homo sapiens]	26	15	85.5	7.06	1.106	0.992505
Q92692-1	Nectin-2 [OS=Homo sapiens]	16	7	57.7	4.82	1.105	0992505

P09564	T-cell antigen CD7 [OS=Homo sapiens]	10	2	25.4	7.27	1.103	0.992505
P01700	Immunoglobulin lambda variable 1-47 [OS=Homo sapiens]	25	2	12.3	5.91	1.105	0.992505
P19438-5	Isoform 5 of Tumor necrosis factor receptor superfamily member 1A [OS=Homo sapiens]	14	2	24.2	7.71	1.104	0.992505
P02533	Keratin, type I cytoskeletal 14 [OS=Homo sapiens]	34	13	51.5	5.16	1.037	0.993555
P10253	lysosomal alpha-glucosidase [OS=Homo sapiens]	39	23	105.3	6	1.046	0.997917
P02790	Hemopexin [OS=Homo sapiens]	60	19	51.6	7.02	1.08	0.997917
P01008	Antithrombin-III [OS=Homo sapiens]	37	14	52.6	6.71	1.047	0.997917
Q6EMK4	vasorin [OS=Homo sapiens]	25	11	71.7	7.39	1.06	0.997917
P35555	Fibrillin-1 [OS=Homo sapiens]	4	10	312	4.93	1.096	0.997917
P21796	voltage-dependent anion-selective channel protein 1 [OS=Homo sapiens]	30	7	30.8	8.54	1.097	0.997917
P02748	complement component C9 [OS=Homo sapiens]	17	7	63.1	5.59	1.056	0.997917
P08195-4	Isoform 4 of 4F2 cell-surface antigen heavy chain [OS=Homo sapiens]	13	6	71.1	4.97	1.055	0.997917
Q8TDQ0	Hepatitis A virus cellular receptor 2 [OS=Homo sapiens]	26	6	33.4	5.72	1.083	0.997917
O43895	Xaa-Pro aminopeptidase 2 [OS=Homo sapiens]	10	5	75.6	6.04	1.08	0.997917
P62979	Ubiquitin-40S ribosomal protein S27a [OS=Homo sapiens]	30	4	18	9.64	1.062	0.997917
Q6GTX8	Leukocyte-associated immunoglobulin-like receptor 1 [OS=Homo sapiens]	13	4	31.4	5.63	1.063	0.997917
P38606	V-type proton ATPase catalytic subunit A [OS=Homo sapiens]	8	3	68.3	5.52	1.096	0.997917
Q7Z7M0	Multiple epidermal growth factor-like domains protein 8 [OS=Homo sapiens]	2	3	302.9	6.87	1.055	0.997917
Q9HBB8-1	Cadherin-related family member 5 [OS=Homo sapiens]	6	3	88.2	4.93	1.049	0.997917
P04040	catalase [OS=Homo sapiens]	5	2	59.7	7.39	1.086	0.997917
P01824	immunoglobulin heavy variable 4-39 [OS=Homo sapiens]	20	2	13.9	9.26	1.092	0.997917
P10909-2	Isoform 2 of Clusterin [OS=Homo sapiens]	27	13	57.8	6.68	1.072	0.998298
Q14315	Filamin-C [OS=Homo sapiens]	1	2	290.8	5.97	1.066	0.998298
Q08380	Galectin-3-binding protein [OS=Homo sapiens]	34	16	65.3	5.27	1.067	0.998434
P13598	Intercellular adhesion molecule 2 [OS=Homo sapiens]	15	3	30.6	7.43	1.071	0.998434

Q5JS37	NHL repeat-containing protein 3 [OS=Homo sapiens]	18	4	38.3	6.43	1.069	1
Q07837	neutral and basic amino acid transport protein rBAT [OS=Homo sapiens]	4	2	78.8	5.96		
P50053	Ketohexokinase [OS=Homo sapiens]	8	2	32.5	6.32		
P07358	Complement component C8 beta chain [OS=Homo sapiens]	6	2	67	8.13		
P09488	Glutathione S-transferase Mu 1 [OS=Homo sapiens]	14	2	25.7	6.7		